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Timely changes of the circulating concentrations of haptoglobin and leptin in female cattle undergoing fat mobilization: assessment of the relevance of haptoglobin as an adipokine

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Dedicated to

My husband, Dr. Firas Al-Zyoud,

who has always encouraged me to pursue my dreams

Timely changes of the circulating concentrations of haptoglobin and leptin in female cattle undergoing fat mobilization: assessment of the relevance of haptoglobin as an adipokine

This study was undertaken to characterize the concentrations of haptoglobin (Hp) and leptin throughout the entire lactation period, to investigate the effect of long-term conjugated linoleic acids (CLA) supplementation on Hp and leptin in dairy cows, to examine the presence of Hp in bovine adipose tissue (AT), and to examine the effect of moderate feed restriction and re-feeding on Hp and leptin in non-lactating heifers. Samples from four different studies were used in this work. The first experiment "CLA-252 days in milk (DIM)" comprised heifers and cows observed from day (d) 21 ante partum (AP) until d 252 post partum (PP), receiving two doses of rumen-protected CLA supplementation (50 and 100 g/d, d 1 - 182 PP) versus a corresponding control group. A further experiment "CLA-post mortem (PM)-105 DIM" was done in heifers observed from d 21 AP until d 105 PP receiving either no CLA or 100 g/d CLA (d 1 - 105 PP). The CLA supplementation included equal proportions of each the t-10, c-12 and the c-9, t-11 isomer. The third experiment "Concentrate-roughage ratio" was conducted (d 21 AP - d 21 PP) using cows that received diets with either a concentrate-to-roughage ratio (dry matter basis (DM)) of 30:70% (low-concentrate group) or 60:40% (high-concentrate group) after calving. The fourth experiment "Fat heifers restriction" was performed using non-lactating heifers fed either grass silage (100%) or grass silage diluted with straw (37:63 on DM basis). The physiological changes of Hp in the CLA-252 DIM experiment were related to parity and parturition. The concentrations of Hp were higher in heifers than in cows during the first week PP. The concentrations of Hp peaked around calving and decreased afterwards in both parity groups and CLA experiments. Long-term feeding of dairy cows with CLA had no effect on the concentrations of serum Hp. The animals in the concentrate-roughage ratio study had lower concentrations of Hp compared to those in the CLA studies. The immunohistochemistry and Western immunoblotting methods indicated the presence of Hp in bovine AT. The physiological changes in the concentrations of leptin in the CLA-252 DIM experiment were limited to parturition. Long-term feeding of dairy cows with CLA left the concentrations of leptin unaffected. The fat heifers restriction study indicated that neither Hp nor leptin concentrations were affected by the change in energy content of the ration fed. In conclusion, long-term feeding of dairy cows with CLA left the concentrations of Hp and leptin unaffected. The presence of Hp in bovine AT indicates that Hp can be classified as an adipokine in cattle. The lack of an evident relation between Hp and body fatness makes Hp irrelevant as an adiposity marker for ruminants.

Zeitlicher Verlauf der zirkulierenden Konzentrationen von Haptoglobin und Leptin in weiblichen Rindern, die eine Fettmobilisierung durchleben: Einschätzung der Relevanz von Haptoglobin als Adipokin

Diese Studie wurde durchgeführt, um die Blut-konzentrationen von Haptoglobin (Hp) und Leptin über die gesamte Laktation zu charakterisieren, um den Langzeiteffekt der Supplementierung von konjugierten Linolsäuren (CLA) auf Hp und Leptin in Milchkühen zu prüfen und die Anwesenheit von Hp in bovinem Fettgewebe zu untersuchen. Außerdem wurde der Effekt einer moderaten Futterrestriktion mit anschließender Wiederaufnahme der Fütterung auf Hp und Leptin in nicht-laktierenden Färsen ermittelt. Proben aus vier verschiedenen Studien wurden in dieser Arbeit verwendet: Der erste Versuch "CLA-252 DIM" beinhaltete Färsen und Kühe (untersucht von d 21 ante partum (AP) bis d 252 post partum (PP)), die von d 1 - 182 PP mit 0, 50 oder 100 g/d pansengeschützten CLA supplementiert wurden. Ein weiterer Versuch "CLA-post mortem (PM)-105 DIM" wurde mit Färsen durchgeführt; diese wurden von d 21 AP bis d 105 PP untersucht und erhielten 100 g/d CLA oder ein CLA-freies Kontrollsupplement. Die zugefütterten CLA enthielten zu gleichen Teilen das t-10, c-12 und das c-9, t-11 Isomer. Der dritte Versuch "Kraftfutter-Rauhfutter-Verhältnis" (KF:R) (d 21 AP - d 21 PP) wurde mit Kühen durchgeführt, deren Rationen nach der Kalbung, bezogen auf Trockenmasse geringe (KF:R = 30:70%) oder hohe (KF:R = 60:40%) KF-Anteile enthielten. Der vierte Versuch "Fette Färsen" wurde mit nicht-laktierenden Färsen durchgeführt, die entweder mit Grassilage (100%) oder mit durch Stroh verdünnte Grassilage (37:63 bezogen auf Trockenmasse) gefüttert wurden. Die physiologischen Veränderungen von Hp im CLA-252 DIM Versuch standen im Zusammenhang zu Parität und Geburt. Die Hp-Konzentrationen in der ersten Woche nach der Geburt waren bei Färsen höher als bei Kühen. In beiden Paritätsgruppen und CLA-Versuche konnte gezeigt werden, dass die Hp-Konzentration ihr Maximum zum Zeitpunkt der Geburt hatte und anschließend wieder absank. Mit der Langzeitfütterung von CLA konnten keine Veränderungen der Hp-Serum Konzentrationen erkannt werden. Die Tiere des Versuches "KF:R" hatten niedrigere Hp-Konzentrationen im Vergleich zu denen der CLA-Versuche. Durch Immunhistochemie und Western Blot konnte die Anwesenheit von Hp in bovinen Fettgewebe gezeigt werden. Die physiologischen Veränderungen der Leptinkonzentrationen im CLA-252 DIM Versuch waren auf den Zeitraum der Geburt begrenzt. Die CLA-Langzeitfütterung der Milchkühe ließ die Leptinkonzentration unbeeinflusst. Der Versuch mit

fetten Färsen deutete drauf hin, dass weder Hp- noch Leptinkonzentrationen durch die Veränderung des Energiegehaltes des Futters beeinflusst waren. Zusammenfassend konnte gezeigt werden, dass Langzeitfütterung von Milchkühen mit CLA zu keiner Veränderung der Hp- und Leptinkonzentrationen führte. Die Anwesenheit von Hp in bovinem Fettgewebe bestätigt, dass Hp in Wiederkäuern als Adipokin klassifiziert werden kann. Der fehlende Zusammenhang zwischen Hp und Körperfettgehalt disqualifiziert Hp als Marker für den Körperfettgehalt in Wiederkäuern.

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List of abbreviations

ACTH	Adrenocorticotropic hormone
ADF	Acid detergent fiber
AP	Ante partum
APP(s)	Acute phase protein(s)
APR	Acute phase response
APS	Ammonium persulfate
AT	Adipose tissue
BCS	Body condition score
BHB	β-hydroxybutyrate
BW	Body weight
c	cis
CLA	Conjugated linoleic acids
CNS	Central nervous system
COX	Cyclooxygenase
CP	Crude protein
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein
CTR	Control
CV	Coefficient of variation
d	Day
Da	Dalton
DIM	Days in milk
DM	Dry matter
DMI	Dry matter intake
DMSO	Dimethyl sulfoxide
DTT	Dithiothreitol
EB	Energy balance
EDTA	Ethylenediaminetetraacetic acid
EE	Ether extract
ELISA	Enzyme-linked immunosorbent assay
FA	Fatty acids
Fig.	Figure

FSH	Follicle stimulating hormone
g	Earth's gravity constant
GH	Growth hormone
GLM	General linear model
GnRH	Gonadotrophin-releasing hormone
Hb	Haemoglobin
HC	High concentrate
Hp	Haptoglobin
HPA	Hypothalamic-pituitary-adrenal
HRP	Horseradish peroxidase
IGF-I	Insulin-like growth factor-I
IHC	Immunohistochemistry
IL-1	Interleukin-1
IL-6	Interleukin-6
LC	Low concentrate
LH	Luteinizing hormone
LOX	Lipoxygenase
ME	Metabolizable energy
MER	Maintenance energy requirement
mRNA	Messenger ribonucleic acid
n	Number
NDF	Neutral detergent fiber
NEB	Negative energy balance
NEFA	Nonesterified fatty acids
NEg	Net energy for gain
NE_L	Net energy lactation
NPY	Neuropeptide Y
ns	not significant
ob	Obese gene
OD	Optical density
P	Probability
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline

PM	Post mortem
PMR	Partial mixed ration
PP	Post partum
PPAR	Peroxisome-proliferator-actived receptor
PPRE	Peroxisome proliferator response element
PUFA	Polyunsaturated fatty acids
PVDF	Polyvinylidene fluoride
r	Correlation coefficient
RA	Retinoid acid
ROC	Receiver operating characteristics
rpm	Rotations per minute
RT	Room temperature
RXR	Retinoid X receptor
SAA	Serum amyloid A
SC	Subcutaneous
SCC	Somatic cell count
SDS	Sodium dodecyl sulfate
sec	Second
SEM	Standard error of the mean
t	trans
Tab.	Table
TBST	Tris-Buffered Saline-Tween
TEMED	Tetramethylenediamine
TMB	Tetramethylbenzidine
TMR	Total mixed ration
TNF	Tumor necrosis factor
VC	Visceral
VLDL	Very low density lipoproteins
vs.	versus

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1. Introduction

Livestock play a vital role in agricultural and rural economies of the world. Not only do they produce food directly but also they provide key inputs to crop agriculture and are a crucial link in nutrient cycles. Cattle are producers of both milk and meat and contribute greatly to human food supply. Over the last 50 years, dairy farming has become more intensive to increase the amount of milk produced by each cow. Dairy breeds such as Holstein Friesian have been selected to produce a very high yield of milk around 55 kg/day (d) (Breves, 2007). However, dairy cattle production exposes animals to a variety of stressors which can reduce feed intake and disease resistance. For instance, 50% of cows have one or more adverse health events during the periparturient period (Ferguson, 2001). During this period, the energy requirements can not entirely be met through dietary intake resulting in a condition of negative energy balance (NEB), as a consequence extensive metabolic and endocrine changes occur in dairy cows to accommodate parturition and lactogenesis (Bell, 1995). High-yielding dairy cows are then susceptible to various metabolic diseases, impaired immune function and fertility (Butler and Smith, 1989; Mallard et al., 1998). Dairy cattle production has been looking for more efficient and effective ways to minimize the health problems for a long time. Therefore, dietary adjustments that could enhance homeostasis and health status are given a broader scope (Bassaganya-Riera et al., 2001). In the last decade, there was sharply increased interest of the physiological effects of various fatty acids (FA), in particular to investigate the conjugated linoleic acids (CLA), which are naturally included in the milk and meat of ruminants, due to the potentially beneficial effects in terms of animal welfare, nutritional status and diseases. Besides the nutritional strategies, measurement of acute phase proteins (APPs) and hormonal concentrations can also be used to enhance cattle health and welfare status.

1.1. Transition period

The transition or periparturient period, beginning three weeks before calving and ending three weeks post-calving, clearly is the most critical phase of the lactation cycle for dairy cows (Grummer, 1995). This period is associated with elevated incidence of diseases such as mastitis, milk fever, metritis, ketosis, retained fetal membranes and displaced abomasum (Mulligan and Doherty, 2008). During this time, transition cows experience alterations related to increases in energy demands driven by both fetal growth requirements and lactogenesis

(Bell, 1995), and alterations related to metabolic, physical, behavioral and hormonal changes around parturition which are marked by a reduction in dry matter intake (DMI) (Allen et al., 2005). Consequently, cows enter a situation of NEB. However, a significant adaptation to NEB is the mobilization of fat from body stores with release of nonesterified fatty acids (NEFA) into blood. In muscular tissue, proteolysis is increased and the mobilized amino acids contribute as substrates in the gluconeogenesis and milk protein synthesis (Herdt, 2000). The concentration of NEFA in blood reflects the degree of adipose tissue (AT) mobilization (Pullen et al., 1989). When the concentration of NEFA in blood increases around calving or in early lactation, more NEFA are taken up by the liver (Reynolds et al., 2003). In the liver, NEFA can be (1) completely oxidized to carbon dioxide to provide energy for the liver; (2) partially oxidized to produce ketone bodies such as β-hydroxybutyrate (BHB) that are released into the blood and serve as fuels for other tissues; or (3) reconverted to triglycerides (Drackley, 1999). A part of the triglyceride produced is exported out of the cells in the form of lipoproteins, of which very low density lipoproteins (VLDL) constitute the largest part (Drackley, 1999). If NEFA uptake by the liver becomes excessive, fatty liver may develop when the synthesis of triglycerides exceeds hepatic export capacity (Bobe et al., 2004). Negative energy balance and glucose deficit after calving also lead to increased production of BHB, which can result in clinical or subclinical ketosis (Herdt, 2000). Thus, increased circulating NEFA and BHB are highly associated with periparturient disorders and diseases (Herdt, 2000; Bobe et al., 2004). Moreover, the higher concentration of NEFA around calving could be related to inflammation (Kováč et al., 2009); thereby increasing susceptibility to inflammatory diseases including mastitis and metritis. Elevated plasma NEFA and BHB concentrations are usually accompanied by a reduction of plasma glucose, insulin and growth hormone (GH) concentrations, which reflects the greater glucose demand with the onset of lactation.

1.2. Adipose tissue

Adipose tissue is a type of loose connective tissue comprised of lipid-filled cells termed as adipocytes. Besides adipocytes, it contains connective tissue matrix, nerve cells, stromovascular cells (e.g. preadipocytes, fibroblast-like cells), and immune cells (Frayn et al., 2003). Adipose tissue provides a virtually limitless storage site for energy in form of triglycerides, which are mobilized through lipolytic pathways to provide fuel to other organs. There are two types of AT existing in mammals: brown and white AT. Brown AT contains

several small lipid droplets and high numbers of mitochondria, its main role is to generate body heat particularly in neonates and hibernating species (Tran and Kahn, 2010). White AT, the predominant type of AT, is characterized by spherical adipocytes with a single lipid inclusion, eccentrically located nucleus and a small volume of cytoplasmic material at the cell periphery (Shen et al., 2003). However, only the white AT will be further addressed in this work. White AT is an active endocrine organ that secretes a variety of bioactive molecules called adipokines or adipocytokines. These adipokines include hormones implicated in energy balance (EB) (e.g. leptin, adiponectin), glucose tolerance and insulin sensitivity (e.g. adiponectin, resistin), cytokines (e.g. Interleukin-6 (IL-6), tumor necrosis factor-α (TNF-α)), and proteins involved in lipid metabolism (e.g. retinol binding protein, cholesteryl ester transfer protein), vascular homeostasis (e.g. plasminogen activator inhibitor-1, angiotensinogen), and in inflammation and stress responses (haptoglobin (Hp), metallothionein) (Trayhurn and Wood, 2004). White AT is consisting of different depots which are located in specific regions of the body. These depots are categorized into subcutaneous (SC) and internal AT. The SC depot comprises the fat layer found between the dermis and the aponeuroses and fasciae of the muscles, and anatomically is subdivided into superficial and deep SC AT (Shen et al., 2003). The internal AT is located around the internal organs, divided into visceral (VC; e.g. intrathoracic, intraabdominopelvic (e.g. omental, mesenteric, retroperitoneal)), and non-visceral (distributed among muscles and adjacent to bones) fat (Shen et al., 2003).

The AT depots are different in terms of their metabolic functions according to the anatomic location of each depot, e.g. in human obesity, VC depots (omental) show a stronger connection with metabolic disorders (e.g. insulin resistance, type 2 diabetes), while SC depots are associated with improved insulin sensitivity and a lower risk of developing type 2 diabetes mellitus (Pérez-Pérez et al., 2009; Tran and Kahn, 2010). Subcutaneous AT secretes adiponectin more abundantly than VC AT (Nakamura et al., 2009), whereby this adipokine is associated with improvement of insulin sensitivity (Kim et al., 2007). In contrast, resistin and retinol-binding protein are associated with insulin resistance and type 2 diabetes mellitus, which are more abundantly secreted from VC than SC AT (Klöting et al., 2007). The omental but not the SC fat drains into the portal vein, and releases excess NEFA which interferes with the liver metabolism and contributes to glucose intolerance and other metabolic complications associated with human obesity (Pérez-Pérez et al., 2009). The metabolic differences among depots are also reported in cattle. In this regard, Hishikawa et al. (2005) reported that

adipocytes in the SC and VC depots showed differences in their basal metabolic properties such as regulating volume and lipid composition; also they present differential gene expression profiles.

1.3. Immune system

Animals undergo challenges to their state of health, and are protected by the immune system that consists of a complex network of cells and soluble mediators. The immune system is classified into two categories: innate and adaptive (acquired) immunity, which are highly interactive and coordinated to provide an optimal resistance to infectious diseases. Innate immunity is not specific for a particular antigen. The innate immune response is the first and fast-acting line of host defense against the invading pathogens; this response can be localized within affected tissues or mobilized to the site of infection (systemic). Components of the innate defense include the physical barrier of the skin and mucous epithelia, leukocytes (macrophages, neutrophils and natural killer cells), non immune cells (epithelial and endothelial cells), and certain soluble mediators (cytokines, eicosanoids and APPs). Acquired or specific immunity is triggered if the pathogen is able to evade or is not completely eliminated by the innate defense system, and it involves cells called lymphocytes (B and T cells). If the host should encounter with the same antigen more than once, immune reactivity will occur as a consequence of the immunological memory. Inflammation is one of the hallmarks and first responses of the innate immune system to infection, and is associated with heat, redness, pain, swelling and impaired function. It has two main functions in order to remove the injurious agents and to initiate the tissue healing process. The above mentioned section is summarized according to Lippolis (2008) and Sordillo et al. (2009). The local inflammation that develops at the site of infection induces the acute phase response (APR).

1.4. Acute phase response and acute phase proteins

The APR is a complex systemic innate-defense system activated by trauma, infection, stress and inflammation to prevent tissue damage, eliminate any infective organisms and activate the repairing processes in order to restore homeostasis. It is induced by the release of inflammatory cytokines, especially Interleukin-1 (IL-1), IL-6 and TNF-α from the macrophages or blood monocytes at the site of inflammatory lesions or infections. These inflammatory mediators set of both the local effects on adjacent cells (leukocytes, fibroblasts, smooth muscle cells and endothelial cells), and systemic effects on other organs that can be

reached through the blood stream. Liver is the main site of synthesis of most APPs. Cytokines therefore act like mediators between the local site of injury and the hepatocytes (liver) to produce and release the APPs. Cytokines can also act indirectly on the APPs production via the hypothalamic-pituitary-adrenal (HPA) axis, which involves the secretion of corticotropin-releasing hormone (CRH) and the adrenocorticotropic hormone (ACTH), and subsequent production of cortisol. However, IL-6 is the major stimulator for the hepatocytic secretion of most APPs. The APPs are defined as proteins whose plasma concentrations increase or decrease classifying them into positive (e.g. C-reactive protein (CRP), serum amyloid A (SAA), Hp), and negative (e.g. albumin, transferrin, transcortin) APPs, respectively. The maximum concentrations are usually reached within 24 - 48 h after stimulation and decline with recovery from the infection. The above mentioned section is based on the review of Jain et al. (2011). Acute phase proteins' changes reflect the presence and intensity of inflammation, and they have long been used as a clinical guide for diagnosis (Petersen et al., 2004). The production of APPs differs among species with regard to their increase in concentrations in response to stimuli. In humans, CRP and SAA showed the highest increases during an APR (Jain et al., 2011). C-reactive protein, Hp and pig major APP (pig-MAP) are major APPs in pigs (Carpintero et al., 2005). In cattle, there are two major APPs, Hp and SAA which both increase during tissue injury and disease (Grönlund et al., 2005). This work will mainly focus on Hp as one of the strongly reacting APPs in cattle.

1.4.1. Haptoglobin

1.4.1.1. Structure and phenotypes

Haptoglobin is an α_2-glycoprotein with haemoglobin (Hb)-binding capacity, originally synthesized as a single αβ polypeptide which is cleaved by a protease into α and β chains, and then covalently linked via disulfide bonds producing the mature Hp (Kurosky et al., 1980). Glycosylation is an enzymatic process that attaches glycans to proteins, lipids or other organic molecules, which regulates the configuration and function of glycoproteins. For instance, human Hp is a liver-secreted serum glycoprotein with four potential N-glycosylation sites on its β chain (Shu et al., 2011). However, there are two major α chain sub-units; a shorter (α_1) and a longer (α_2) variation. The α_1 and α_2 chains form a link to one and two other α chains, respectively. The Hp gene is characterized by two common alleles, Hp 1 and Hp 2. The two alleles give rise to three different phenotypes (Fig. 1), which are composed of two $\alpha_1\beta$ units

(in Hp 1-1), two $\alpha_1\beta$ and variable numbers of $\alpha_2\beta$ units (in Hp 2-1), or variable numbers of $\alpha_2\beta$ units (in Hp 2-2) (Cheng et al., 2007; Lai et al., 2008). Although Hp is found in serum of all mammals, this polymorphism (Hp 1-1, Hp 2-1 and Hp 2-2) exists only in humans, while it has been suggested that the structure of Hp in ruminants is similar to human Hp 2-2 (Lai et al., 2008). Bovine Hp consists of multiple α and β subunits with respective molecular weights reported as 16 kDa (α-chains) and 40 kDa (β-chains) (Eckersall and Conner, 1990) or 20 kDa peptide (α-chains) and 35 kDa glycopeptide (β-chains) (Morimatsu et al., 1991). In circulation, it is highly polymerized having a molecular weight of approximately 1000-2000 kDa (Godson et al., 1996).

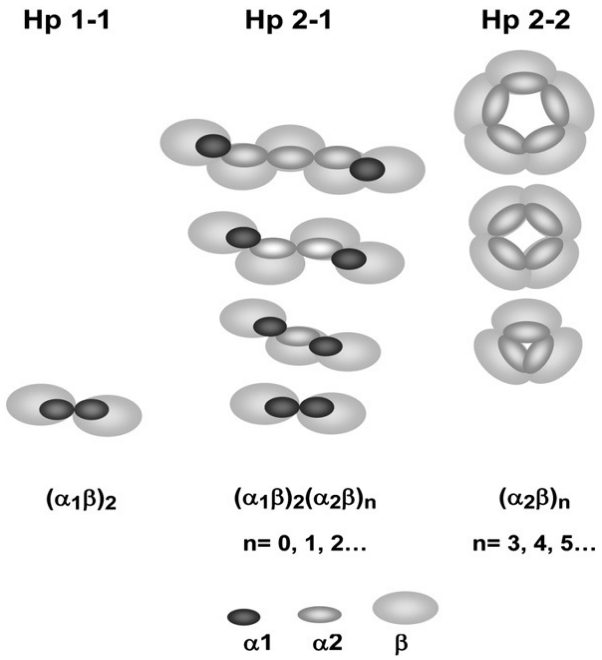

Fig. 1. A schematic model illustrating the structure of human Hp phenotypes. Hp 1-1 represents a homodimer, the simplest combination of dimeric $\alpha_1\beta$ chains $(\alpha_1\beta)_2$. Hp 2-2 is heterogeneous in size, starting with cyclic trimeric $\alpha_2\beta$ chains $(\alpha_2\beta)_3$ and other cyclic polymers. Hp 2-1 is also heterogeneous, but composed of simple homodimer $(\alpha_1\beta)_2$, a linear trimeric αβ chain $(\alpha\beta)_3$, and other linear polymers; where α represents a mixture of α_1 and α_2 chains. All types share a common structure of the β chain (Cheng et al., 2007).

1.4.1.2. Sites of production

Haptoglobin is mainly produced by the liver and secreted into the serum. Besides blood serum, Hp is found in bovine milk (Hiss et al., 2004), in human cord serum, cerebrospinal fluid, amniotic and vaginal fluids (Katnik and Dobryszycka, 1990), and in porcine saliva (Hiss et al., 2003). Haptoglobin messenger ribonucleic acid (mRNA) expression has been

reported under normal and inflammatory conditions in a variety of organs and tissues of mice including lungs, skin, spleen and kidneys (D'Armiento et al., 1997), adrenal gland, ovary and uterus (Friedrichs et al., 1995). For cattle, Hp mRNA is reportedly expressed in mammary gland (Hiss et al., 2004; Thielen et al., 2005), oviduct and liver (Lavery et al., 2004), and leukocytes (Thielen et al., 2005). There is a growing body of evidence indicating that Hp mRNA is expressed in AT. It was suggested that Hp is also involved in obesity because its serum concentrations are increased in obese humans (Chiellini et al., 2004), and this is consistent with the concept that obesity is a state of chronic mild inflammation (do Nascimento et al., 2004). In this regard, Friedrichs et al. (1995), Chiellini et al. (2002) and do Nascimento et al. (2004) reported that Hp gene is expressed in AT in normal and obese mice. Similarly, Hp gene expression has also been identified in human AT (do Nascimento et al., 2004). The above mentioned authors concluded that Hp synthesis occurs in adipocytes rather than in other cell types within AT. Moreover, there are differences among the different AT depots in terms of Hp expression, i.e. VC depots release more Hp than SC depots in humans (Fain et al., 2004).

1.4.1.3. Physiological functions

The main function of Hp is binding Hb to prevent losses of iron via urine after haemolysis, thereby protecting tissues from being damaged by free Hb (Langlois et al., 1996; Petersen et al., 2004). The Hp-Hb complex has several other benefits: (1) it restricts the availability of iron necessary for bacterial growth, and thus inhibits bacterial activity (Delanghe et al., 1998); (2) it scavenges the Hb-driven free radicals (superoxide (O_2^-) and hydroxyl (OH)) during oxidative stress (Melamed-Frank et al., 2001); (3) it inhibits nitric oxide, which is a potent vasodilator molecule that is produced by vascular endothelial cells (Edwards et al., 1986); and (4) it inhibits eicosanoid (prostaglandin) synthesis (Komoriya et al., 1980), hence Hp is suggested as an anti-inflammatory agent. Haptoglobin also exhibits other activities. For instance, Hp has been identified as a strong angiogenic agent required for proliferation and differentiation of endothelial cells in the formation of new blood vessels (Park et al., 2009), and as a modulator of the immune system, i.e. it inhibits the activation of neutrophils (Oh et al., 1990). Haptoglobin in AT might act as a monocyte chemoattractant factor (Maffei et al., 2009), and as an antioxidant or angiogenesis agent (do Nascimento et al., 2009).

1.4.1.4. Haptoglobin as an indicator for health status

In cattle and other ruminants, Hp is suggested as a marker of inflammation. In healthy cattle, Hp blood concentrations are low or even undetectable (Eckersall et al., 2001), but increase over 1000-fold (Eckersall et al., 2006) upon immune stimulation. For milk, a 160-fold increase in Hp concentrations has been observed after intramammary challenge with lipopolysaccharide (Hiss et al., 2004). Several studies have indicated the significance of Hp in serum for identifying the occurrence and severity of inflammatory responses such as mastitis (Eckersall et al., 2006; Hiss et al., 2007), and metritis (Chan et al., 2004; Huzzey et al., 2009). Elevated Hp concentrations occur with some conditions not generally associated with inflammation or tissue damage such as fatty liver (Nakagawa et al., 1997), and stress associated with transportation for 2 days (Murata and Miyamoto, 1993). Elevated Hp concentrations were also observed after surgical castration in bull calves (Fisher et al., 2001), and tail docking in heifers (Eicher et al., 2000). Several studies have been conducted to define the cut-off values for serum Hp to discriminate between healthy and diseased animals. In this regard, a cut-off value for serum Hp of 0.15 mg/mL was defined in dairy heifer calves with respiratory-tract disease, whereby sensitivity and specificity were 72% and 59%, respectively (Svensson et al., 2007). A cut-off value of 0.05 mg/mL was used to differentiate between healthy cows and those with mastitis, whereby sensitivity and specificity were 83% and 90%, respectively (Eckersall et al., 2001). In milk, to distinguish between healthy quarters and those with subclinical mastitis a cut-off value of 2.2 µg/mL was determined by Hiss et al. (2007), whereby sensitivity and specificity were 85% and 92%, respectively.

1.5. Leptin

1.5.1. Structure

The product of the obese gene (ob), which was discovered in the mid-1990s by Zhang et al. (1994), was named leptin (derived from the Greek term *leptos* meaning thin). Leptin, a 16-kDa protein consisting of 146 amino acids is released into the blood after cleavage of the 21 amino acid signal peptide (Zhang et al., 1994). Leptin is a four-helix protein and contains a short helical segment (Fig. 2), which is similar to the structure of the cytokine-family (Zhang et al., 1997). Leptin contains one single disulfide bond that links two cysteines (Fig. 2), and

this bond has been proven critical for the structural integrity and stability of leptin (Rock et al., 1996).

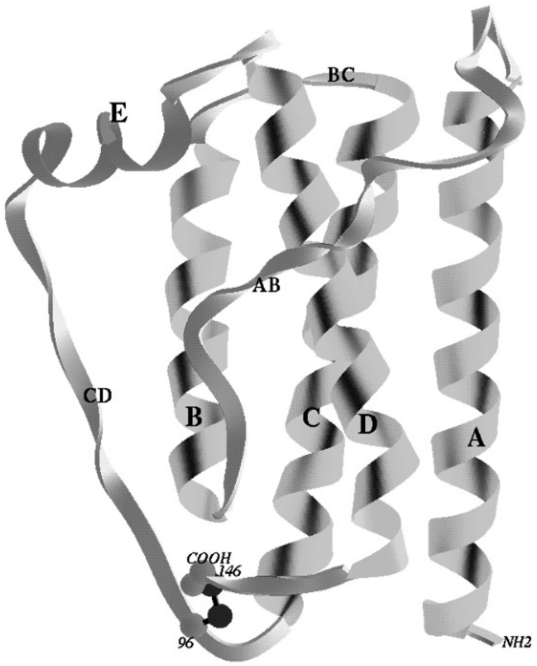

Fig. 2. Ribbon diagram of the ob protein. The view is perpendicular to the four helical bundle axis (A, B, C and D), the additional short helical segment (E), and the connecting loops AB, BC and CD. The N and C terminal are indicated. The disulphide bridge between the C-terminal Cys 146 and Cys 96 in the AB loop is represented with ball-and-stick model (Zhang et al., 1997; Liefers, 2004).

1.5.2. Occurrence and sites of production

Leptin is an adipose-derived hormone that regulates a wide variety of physiological processes including appetite and EB, and it is also involved in the regulation of reproduction, neuroendocrine and immune function (Ahima and Flier, 2000; Ingvartsen and Boisclair, 2001). Despite the evidence that leptin is produced mainly by adipocytes, other sites of it's gene expression have been reported such as mammary gland (Casabiell et al., 1997), placental and fetal tissues (Masuzaki et al., 1997; Hoggard et al., 1998), stomach (Bado et al., 1998), muscles (Wang et al., 1998), hypothalamus and pituitary gland (Morash et al., 2000) and brown AT (Cinti et al., 1997). Leptin expression is influenced by the status of energy stores, but other metabolic and endocrine factors participate in regulating leptin production. Leptin synthesis is stimulated by obesity, insulin (Saladin et al., 1995), glucocorticoids (Slieker et al., 1996), infection, endotoxins, cytokines (Grunfeld et al., 1996), glucose, lipids and estrogens (Wang et al., 1998), whereas it is inhibited by thyroid hormones (Escobar-Morreale et al., 1997), fasting, cold, physical activity and testosterone (Castracane et al., 1998).

1.5.3. Role of leptin in food intake and body weight regulation

Leptin is primarily produced in the AT as mentioned before, and acts via a family of membrane bound receptors. A total of six leptin receptor isoforms in different tissues was reported. One isoform with a long intracellular domain (ob-Rb) is predominantly expressed in the hypothalamic regions, and is involved in the regulation of food intake and energy homeostasis (Elmquist et al., 1998). Within the central nervous system (CNS), the hypothalamus is the main site of leptin action with respect to control food intake and energy expenditure (Ingvartsen and Boisclair, 2001). Therefore, leptin must enter the brain cavity to act on the CNS. Most of the plasma proteins enter the brain via the blood-cerebrospinal fluid barrier at the blood-brain barrier at the cerebral endothelium (Pardrige, 1998). Neuropeptide Y (NPY) is secreted by the hypothalamus and regulates food intake; leptin binds to ob-Rb receptors that are mainly localized on NPY neurons and thereby inhibits the signaling of NPY, resulting in a reduction of feed intake and an increase of energy expenditure (Jang et al., 2000). Further, plasma leptin concentrations are affected by changes in the plane of nutrient supply. In sheep, feeding restricted to 39% of the maintenance energy requirements (MER) for 3 d (Delavaud et al., 2000), and to 38% of the MER for 14 weeks (Morrison et al., 2001) decreases plasma leptin concentrations. Complete food deprivation causes a rapid fall in plasma leptin within 24 h in sheep (Marie et al., 2001). In contrast, increasing the dietary intake from moderate to high in pregnant ewes and adult rams increases the concentrations of plasma leptin (Blache et al., 2000; Thomas et al., 2001). In humans and rodents, leptin is synthesized in proportion to the overall degree of adiposity (Friedman and Halaas, 1998). Similar relationships have been observed between leptin and body fatness in sheep (Blache et al., 2000; Thomas et al., 2001; Altmann et al., 2006), body condition score (BCS) (Ehrhardt et al., 2000; Delavaud et al., 2002) and adipocyte size in cows (Delavaud et al., 2002).

1.5.4. Role of leptin in reproduction

Leptin is involved in the regulation of reproductive functions; it stimulates the production of the gonadotropins (luteinizing hormone (LH) and follicle stimulating hormone (FSH)) from the hypophysis via gonadotrophin-releasing hormone (GnRH) neurons in the hypothalamus (Amstalden et al., 2003). Since leptin is involved in the regulation of food intake, body weight (BW) and reproductive function, it is an interesting hormone to be investigated during the periparturient period in dairy cattle when many changes take place both in energy metabolism

and reproductive physiology. During pregnancy, leptin concentrations are high, and then decline rapidly towards parturition (Holtenius et al., 2003; Hachenberg et al., 2007; Duske et al., 2009). These high concentrations of leptin during pregnancy are due to an increase in body fat reserves and a concomitant increase in leptin mRNA expression in AT (Ehrhardt et al., 2001). The decline in plasma leptin concentrations towards parturition with the onset of NEB is probably caused by the inhibition of leptin expression in AT (Block et al., 2001), and mobilization of AT (Liefers et al., 2005). Another reason is the regulation of leptin expression by insulin and GH. The onset of NEB around parturition is also associated with decreased plasma insulin and increased plasma GH (Block et al., 2003), suggesting that both hormones could mediate a portion of the effect of EB on plasma leptin. As mentioned before, leptin is involved in the regulation of immune function, therefore the reduction in leptin concentrations of early lactating cows could contribute to the development of depressed immunity as suggested in fasted mice that leptin has a specific effect on T lymphocyte responses, differentially regulating the proliferation of naive and memory T cells (Lord et al., 1998). Furthermore, leptin increases helper T-cell type 1 and suppresses helper T-cell type 2 cytokine production (Lord et al., 1998). In addition, changes in leptin concentrations of early lactating cows could participate in the co-ordination of metabolism through promoting a faster increase in voluntary feed intake (Block et al., 2001; Holtenius et al., 2003).

1.6. Nutritional considerations during the transition period

Most of the attempts to improve the energy status and minimize the extent and the duration of NEB have been approached by increasing the energy density of the diet by feeding dietary fat sources and increasing non-fiber carbohydrates (e.g. grain-based supplements) (Grummer, 2007). Supplemental fats have proven to be beneficial for improving energy intake and reducing an energy deficit in early lactation. Moreover, feeding supplemental fat could reduce FA mobilization from AT and potentially reduce the incidence of ketosis (Kronfeld, 1982), improve the reproductive efficiency (Staples et al., 1998), and modulate the immune reaction (Calder et al., 2008). In contrast, fat feeding can often result in a depression of DMI due to the effect of fat on ruminal fermentation and gut motility, the decrease of palatability and the increased rate of FA oxidation in the liver (Allen, 2000). Protecting FA from the degradation by rumen bacteria which will be mentioned later, makes them partially inert in the rumen and sometimes can prevent a depression in DMI (Jenkins and Palmquist, 1984). The duration (long- or short-term) of the exposure to the fat feeding, amount and type of FA differ among

studies. A new interest is directed to CLA, as one of the FA that play a role in the reduction of milk fat percentage during lactation in cows. The following section will provide the current knowledge about CLA and it's interaction with Hp and leptin.

1.6.1. Conjugated linoleic acids

1.6.1.1. Structure, biological and commercial formation of conjugated linoleic acids

Fatty acids are playing a central role in metabolism as energy storing units and major building blocks for cellular membranes. Linoleic acids (C18:2), the major FA in most oilseeds, and linolenic acid (C18:3), the major FA in fresh forages, are considered as essential FA because they cannot be synthesized by mammals or by ruminal microorganisms. Both are polyunsaturated fatty acids (PUFA) in that they have more than one double bond. Chilliard et al. (2000) proposed that 80% of dietary linoleic acids are biohydrogenated in rumen due to the toxicity of PUFA to many rumen microorganisms. One of the biohydrogenation intermediates is CLA. Conjugated linoleic acids refer to a group of PUFA that exist as positional and geometric isomers of linoleic acid (Gudbrandsen et al., 2009). The term conjugated pertains to the chemical bond system, in which two double bonds are linked by a single bond. Further, each of these positional conjugated diene isomers occurs in cis (c) or trans (t) configurations (Aydin, 2005).

The existence of CLA is known since over 70 years. In 1935, it was observed that ultraviolet absorbance at 230 nm of milk fat increased when cattle were driven out to pasture in spring (Booth et al., 1935). However, the recent interest in CLA began with the isolation from meat as an anti-carcinogenic factor in mice and rats (Pariza and Hargraves, 1985). Nine different positional and geometric isomers of CLA are reported by Ha et al. (1989), whereby the (c-9, t-11), (t-10, c-12), (t-9, t-11) and (t-10, t-12) isomers account for more than 89% of total naturally occurring CLA, while the (c-9, c-11), (t-9, c-11), (c-10, c-12), (c-10, t-12) and (c-11, c-13) isomers are minor contributors. Of the isomers, the c-9, t-11 is the predominant natural CLA isomer derived mainly from ruminant meat and milk products (Raff et al., 2008). The chemical structures of linoleic acid, the c-9, t-11 and the t-10, c-12 isomers are shown in Fig. 3. Food lipids from ruminants (dairy products, beef and lamb) contain considerably more CLA than lipids from non-ruminants. Concentrations in dairy products are variable and range from 3 to 9 mg/g fat (MacDonald, 2000). Beef contains 1 - 10 mg/g fat with slightly higher concentrations (4 - 19 mg/g fat) in lamb (Schmid et al., 2006). The concentrations in fats from non-ruminants and vegetable oils typically range from 0.6 to 0.9 mg/g fat (Chin et al., 1992).

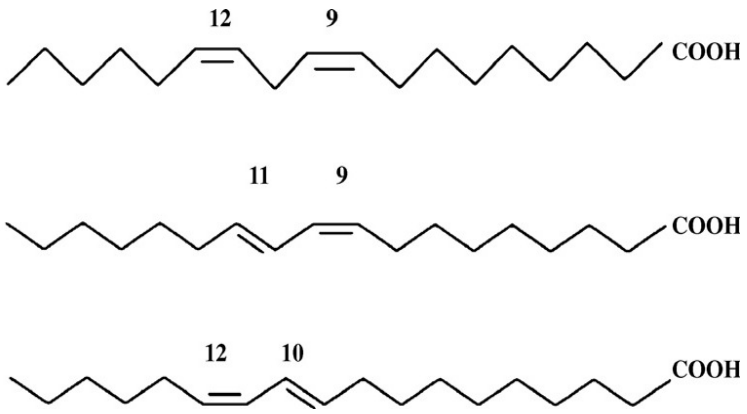

Fig. 3. Structure of linoleic acid (top), c-9, t-11 CLA (mid) and t-10, c-12 CLA (bottom). Double bond positions are indicated by numbers (Aydin, 2005).

Two pathways are contributing to CLA biosynthesis (Fig. 4). The first pathway is the formation of CLA during the ruminal biohydrogenation of linoleic acid, where the sequential reduction steps convert linoleic acid (C18:2 c-9, c-12) to the c-9, t-11 CLA, then to vaccenic acid (C18:1, t-11) and eventually to stearic acid (C18:0) by a rumen microorganism, i.e. by *Butyrivibrio fibrisolvens* (Bauman et al., 2000). The second synthetic pathway arises when the dietary supply of unsaturated FA is high, or the biohydrogenation process may be incomplete. Conjugated linoleic acid can then escape the rumen and become available for absorption in the lower digestive tract, thus providing a source of CLA to the mammary gland and AT through desaturation of C18:1, t-11 by Δ^9-desaturase (Griinari and Bauman, 1999).

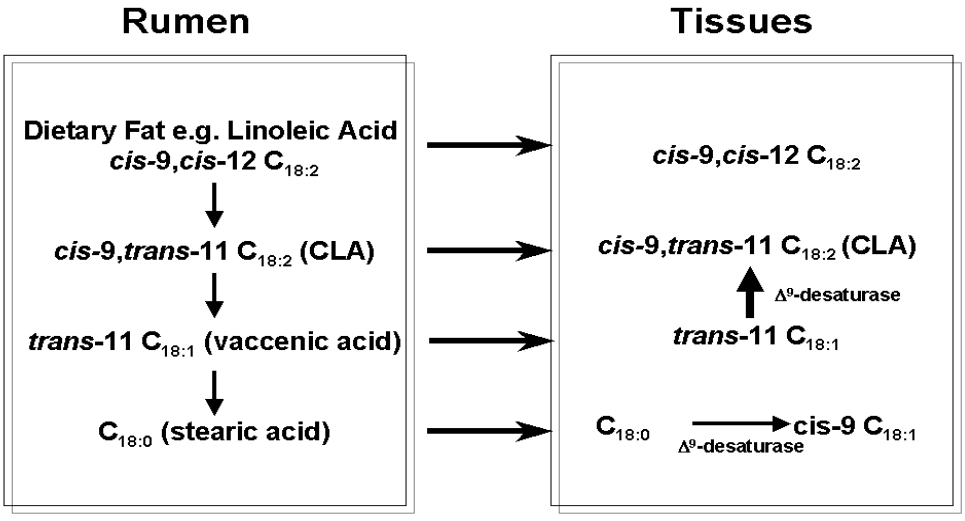

Fig. 4. Role of rumen biohydrogenation and tissue Δ^9-desaturase in the production of c-9, t-11 conjugated linoleic acid in ruminant fat (Bauman et al., 2000).

The commercial CLA supplements are isomeric mixtures usually containing the t-10, c-12 and the c-9, t-11 isomers in equal amounts. The CLA are commercially manufactured by: (1) alkali isomerization of linoleic acid (Yang et al., 2002); (2) dehydration of ricinoleic acid methyl ester (Body and Shorland, 1965); and (3) microbial synthesis of c-9, t-11 from linoleic acid using cultures of different microorganisms (Pariza and Yang, 1999). Several techniques are used to protect fat supplements from the degradation by rumen bacteria. These include the formation of calcium salts, amide linkages, formaldehyde treatment and lipid encapsulation (Moon et al., 2008).

1.6.1.2. Physiological effects of conjugated linoleic acids

A plethora of physiological effects in a wide range of experimental animals has been attributed to CLA including actions as an anti-adipogenic, anti-diabetogenic, anti-carcinogenic, and anti-atherosclerotic factor (Belury, 2002; Wang and Jones, 2004). In addition, CLA affects the rate of bone formation as reported in rats (Li et al., 1999). The beneficial effects of CLA have been extended to include reduction in BW, food and energy intakes, and change in body composition, as well as beneficial regulatory effects on the immune functions. Moreover, CLA supplementation modulates lipid metabolism and leads to milk fat depression in lactating cows (Kay et al., 2007; Bauman et al., 2008). There appears to be an isomer-specific effect of CLA: the t-10, c-12 CLA isomer inhibits bovine milk fat synthesis (Baumgard et al., 2002). This isomer is much more effective at lowering BW and AT mass than the c-9, t-11 CLA isomer in mice (Park et al., 1999). On the other hand, adverse effects of CLA are described in some experimental animals such as inflammatory effects induced by the t-10, c-12 CLA isomer (Poirier et al., 2006), increased fat accumulation in the liver and eventual development of insulin resistance using an equimolar mixture of c-9, t-11 and t-10, c-12 CLA isomer (Tsuboyama-Kasaoka et al., 2000). Special emphasis in the up-coming sections will be given on the effect of CLA on BW and body composition as well as the immune modulatory functions of CLA.

Feeding a mixture of CLA isomers has been reported to reduce BW gain in rats (Ryder et al., 2001) and mice (Park et al., 1999). These studies illustrate that the reduction in BW is due to the action of the t-10, c-12 CLA isomer, whereas others observed no effect on BW (Yamasaki et al., 2003). The ability of CLA to lower AT mass by reducing the accumulation of triglycerides in adipocytes has been demonstrated in humans (Smedman and Vessby, 2001;

Thom et al., 2001), mice (DeLany et al., 1999), and pigs (Ostrowska et al., 1999). Furthermore, CLA induced a reduction in adipocyte size in rats (Poulos et al., 2001), mice (Tsuboyama-Kasaoka et al., 2000), and in dairy cows (Akter et al., 2011). Also, CLA has been reported to modulate adipokine production. For example, circulating leptin is reduced in rats fed 2% CLA (by diet weight; mixed isomers) for 12 weeks (Yamasaki et al., 2000), 1.5% CLA of the diet for 3 weeks (Yamasaki et al., 2003), and 1% CLA (of the diet weight) mixed isomers for 4 weeks (Rahman et al., 2001). Moreover, Parra et al. (2010) reported that when mice received two doses of 3 and 10 mg/d CLA (50:50 blend of c-9, t-11 and t-10, c-12 isomers) for 30 days and twice the amounts for another 35 days, leptin concentrations were decreased with increasing the CLA dose associated with reductions in fat accumulation, NEFA and adiponectin. In humans, 3 g/d CLA (mixed isomers) for 64 days cause a reduction in leptin concentrations without any remarkable change in fat mass (Medina et al., 2000). Conversely, several studies reported that dietary CLA supplementation had no effect on leptin concentrations in beef heifers fed 2% (of total ration on dry matter (DM) basis) rumen-protected CLA salts (a mixture of Ca-salts of palm oil FA with 31% CLA (27.2% c-9, t-11; 32.8% t-10, c-12; 10.6% t-8, c-10; 18.95% c-11, t-13 and 10.5% various t, t CLA isomers)) on the last 32 or 60 days before slaughter (Gillis et al., 2004b), and in dairy cattle in mid to late lactation receiving 10 g/d abomasal infusions of either c-9, t-11 or t-10, c-12 CLA isomer for 5 days (Baumgard et al., 2002).

The mechanisms by which the CLA reduce adiposity are not only attributed to the reduction in food and energy intakes, but also involve other pathways including increasing the metabolic rate, energy expenditure, lipolysis and fat oxidation, and decreasing lipogenesis (Wang and Jones, 2004). Peroxisome-proliferator-activated receptors (PPAR) are a group of nuclear receptor proteins that function as transcription factors regulating the expression of genes and playing essential roles in the regulation of cellular differentiation, inflammation and metabolism (Kersten, 2002). Three different PPAR isotypes were identified: α, β and γ. However, the reasons for the effect of CLA on leptin could be that CLA act as a ligand that activates PPARγ (Medina et al., 2000), which might then reduce leptin gene expression. Conjugated linoleic acids are incorporated into the phospholipid fraction of cell membrane and might thereby have effects on signal transducing pathways and modify leptin production (Medina et al., 2000).

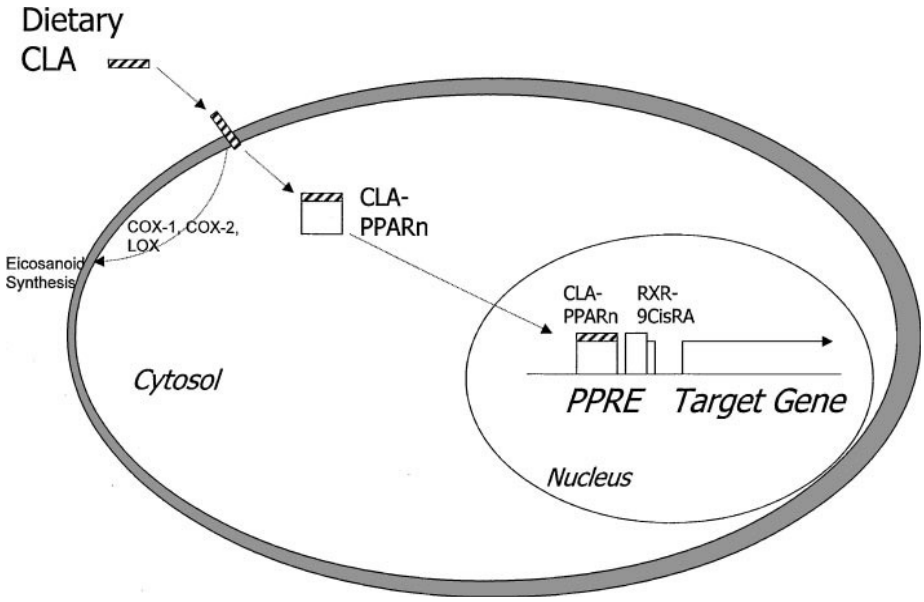

Fig. 5. Schematic diagram of putative cellular and molecular mechanisms of conjugated linoleic acids in modulating systemic conditions such as adiposity and inflammation (Belury, 2002). Abbreviations: COX = cyclooxygenase, LOX = lipoxygenase, PPRE = peroxisome proliferator response element, RXR = retinoid X receptor, 9CisRA = retinoic acid, n = α, β, γ.

Fatty acids are important modulators of the immune system (Calder, 2008; Serhan et al., 2008). In a number of different animals, CLA has been suggested to have anti-inflammatory and/or immune-ameliorating effects mediated partly by PPARγ (Yu et al., 2002). One of the perceived mechanisms by which CLA exerts its health effects in animals is through modulating the formation of eicosanoids through cyclooxygenase (COX) and lipoxygenase (LOX) pathways (Fig. 5), thus exerting a complex control mainly in inflammation and regulation of cytokine synthesis (Belury, 2002). Aspects of both the innate and the adaptive immune responses are affected by dietary CLA supplementation. O'Shea et al. (2004) reported that CLA decreased TNF-α and IL-6, and the c-9, t-11 and t-10, c-12 CLA isomers exerted distinct effects on T cell populations and immunoglobulin subclasses in rats. However, there is limited supporting information about the effect of CLA on APPs. In this regard, Noto et al. (2007) showed that rats fed 1.5% (1.5 g/100 g of total ration) CLA isomers (t-10, c-12; c-9, t-11; c-11, t-13 and t-8, c-10) for 8 weeks had lower serum Hp concentrations and adipose TNF-α mRNA, but no difference in liver Hp mRNA abundance was detected. However, on other inflammation markers, it has been shown that 3.4 g/d CLA (purified t-10, c-12) in men with metabolic syndrome for 12 weeks increase significantly CRP (2.2-fold), but

no significant increase was found for TNF-α and IL-6 (Risérus et al., 2002). In non obese humans, 3.2 g/d CLA (equal amounts of c-9, t-11 and t-10, c-12 isomers) for 12 weeks increased CRP, but CLA supplementation did not have any significant effect with regard to plasma concentrations of TNF-α (Smedman et al., 2005). In contrast, Moloney et al. (2004) found no effect of 3 g/d CLA (50:50 blend of c-9, t-11 and t-10, c-12) supplemented for 8 weeks on CRP or IL-6 in humans.

1.7. Study objectives

Considering the immuno-compromised situation in early lactation and the comprehensive changes in fat mass during the lactation cycle of dairy cows, management of the transition cows emerged to underpin production and profitability on dairy farms. However, in dairy cows CLA is known to decrease milk fat percentage during lactation and decrease energy output during early lactation. It is still unknown if long-term nutritional supplementation with CLA could stimulate or inhibit the expression of the inflammatory response and adipokines, particularly in ruminant animals. Although Hp has been extensively studied under various inflammatory and non-inflammatory conditions, the effect of long-term CLA supplementation on this protein has not yet been investigated. The liver is reported as the main site of Hp production, but other sites of production were proved such as AT in humans and mice, but, however, less is known in ruminants. Researches on how long-term CLA supplementation may alter circulating leptin concentrations are limited. Finally, as early lactating cows mobilize AT under conditions of NEB, the initial step in non-lactating cows to overcome the energy deficit is the mobilization of an excessive quantity of AT. Thus, the question arises if there is any change in the concentrations of Hp and leptin in non-lactating, non-pregnant cows undergoing fat mobilization.

Bearing in mind the above mentioned circumstances, the current study was undertaken to:

1. Characterize the concentrations of Hp and leptin throughout the entire lactation period or early lactation in both heifers and cows.
2. Investigate the potential effect of long-term CLA supplementation on the concentrations of Hp and leptin in dairy cows.
3. Examine the presence of Hp in bovine AT using immunohistochemistry (IHC) and Western immunoblotting.
4. Examine the effect of moderate feed restriction and re-feeding on the concentrations of Hp and leptin in non-lactating, non-pregnant heifers.

2. Materials and methods

2.1. Animals, experiments and diets

Samples from four different studies were used in this work. The first experiment was conducted to characterize the concentrations of Hp and leptin throughout lactation and to evaluate the effects of two different doses of CLA supplementation. This experiment comprised heifers and cows observed from d 21 ante partum (AP) until d 252 post partum (PP) and was named **"CLA-252 days in milk (DIM)"**. In addition, a further experiment using a CLA supplementation was done in heifers investigated from d 21 AP until d 105 PP. In this experiment, the animals were sequentially slaughtered for collection of post mortem (PM) tissue samples. This experiment was termed **"CLA-PM-105 DIM"**. The third experiment **"Concentrate-roughage ratio"** was conducted (d 21 AP - d 21 PP) using cows fed either low or high concentrate. A fourth experiment **"Fat heifers restriction"** using non-lactating, non-pregnant heifers fed either grass silage or grass silage diluted with straw was performed to examine the effect of moderate feed restriction and re-feeding on the concentrations of Hp and leptin. The ingredients and chemical composition of the animals' diets in the different studies are listed in the appendix A (Tab. A1 **-** A7).

2.1.1. Conjugated linoleic acid studies

All animals' experiments were conducted according to the European Union regulations and were approved by the lower saxony state office for consumer protection and food safety (LAVES, file number 33.11.42502-04-071/07, Oldenburg, Germany), and described in detail by Pappritz et al. (2011) and von Soosten et al. (2011). All animals were housed in group pens in a free stall barn equipped with slatted floors and stalls covered with rubber mattresses at the experimental station of the Friedrich Loeffler Institute, Federal Research Institute for Animal Health, Braunschweig, Germany. Each group pen was equipped with a computerized concentrate and a partial mixed ration (PMR) feeding station (Type RIC, Insentec, B.V., Marknesse, The Netherlands). In addition, all animals were marked with an ear transponder to record the daily individual feed intake. Two experiments were performed; the CLA-252 DIM and the CLA-PM-105 DIM experiments, and all diets used were formulated according to the recommendation of the German Society of Nutrition Physiology (GfE, 2001). The BCS of each animal was recorded by using a five-point system (Edmonson et al., 1989). Milking took

place twice daily and the BW was automatically recorded when leaving the milking parlour. The occurred disease events were recorded during milking by the milking staff or during the daily animals control by the stockmen and/or the veterinarian, and when indicated, the animals were attended by the veterinarian. Abnormal vaginal discharges were classified as metritis. Cows with clinical mastitis were identified by farm personnel based on the presence of abnormal milk (presence of flakes, clots or altered color and viscosity). The somatic cell count (SCC) was measured by infrared milk analyzer (Milkoscan FT 6000 combined with a Fossomatic 5000, Foss Electric, Hillerod, Denmark) (von Soosten et al., 2011). The disease incidence rate was defined as the number of disease event cases divided by the total number of animals during the observation period according to Kelton et al. (1998).

2.1.1.1. CLA-252 DIM experiment

2.1.1.1.1. Treatments and diets

German Holstein-Friesian cows (n = 33) and heifers (n = 16) were investigated from d 21 AP until d 252 of the subsequent lactation. The animals were randomly allocated to three dietary treatments taking into account the mean number of lactation (1.9 ± 0.1), the live weight (627 ± 9 kg), and the milk yield of the previous lactation (5,797 ± 122 kg, 200-d milk yield) for the cows. The dietary treatments were: (1) 100 g/d of control fat supplement (Silafat, BASF SE, Ludwigshafen, Germany; **CTR**: 11 cows and 5 heifers); (2) 50 g/d of rumen-protected commercial CLA supplement (lipid encapsulation method; Lutrell Pure, BASF SE, Ludwigshafen, Germany; **CLA-50**: 11 cows and 6 heifers) and 50 g/d of CTR fat supplement; and (3) 100 g/d of CLA (**CLA-100**: 11 cows and 5 heifers). The CLA supplementation started on d 1 PP and lasted until d 182 PP. In the AP period, all animals were received a diet consisting of PMR (40% grass silage (5.4 MJ NE_L/kg) and 60% corn silage (6.4 MJ NE_L/kg)) on DM basis as *ad libitum* consumption and 2 kg/d concentrate (6.7 MJ NE_L/kg) from the computerized concentrate feeding station (Tab. A1). During the supplementation period, the animals were fed PMR (6.8 MJ NE_L/kg) *ad libitum* consisting of 63% silage and 37% concentrate on DM basis, and additionally each animal received 4 kg concentrate (8.8 MJ NE_L/kg) from the concentrate station containing either 50 or 100 g/d of CLA supplement and 50 g of CTR fat supplement (Tab. A2; Pappritz et al., 2011). During the post-treatment period (d 189 - 252 PP), the animals received only PMR without concentrate. The CLA supplement included 78% FA with a proportion of approximately 12% of the t-10, c-12 and c-9, t-11 CLA

isomers in equal parts. In the CTR fat supplement, these isomers were substituted by a corresponding amount of stearic acid (C18:0). The FA profiles for both of CLA and CTR fat supplements are shown in Tab. A3 (Pappritz et al., 2011; von Soosten et al., 2011). The concentration of the t-10, c-12 CLA isomer in concentrates and silages was calculated based on the analyzed concentrations in these feed components. In the CLA concentrate, the concentration of the t-10, c-12 CLA isomer amounted to 2.25 g/kg DM (Tab. A2; Pappritz et al., 2011). Thus, the CLA-50 and CLA-100 groups had intakes of 4 and 8 g of t-10, c-12 CLA/d, respectively.

2.1.1.1.2. Blood collection

Blood samples were drawn from the *Vena jugularis externa* on d –21, –14, –7, 1, 7, 14, 21, 35, 49, 70, 105, 140, 182, 189, 196, 210, 224, 238, and 252 relative to parturition as shown in Fig. 6. The centrifugation of serum was performed after sampling, and the samples were stored at –80°C until analyzed (Pappritz et al., 2011).

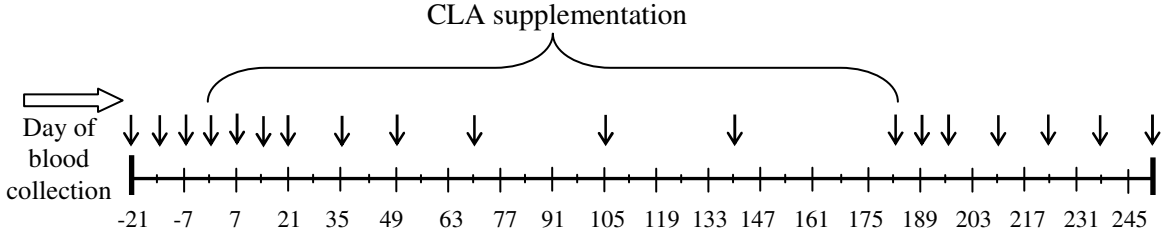

Fig. 6. Diagram of the experimental design and blood collection for the CLA-252 DIM experiment.

2.1.1.2. CLA-PM-105 DIM experiment

2.1.1.2.1. Treatments and diets

German Holstein-Friesian heifers (n = 25) with an average age at first parturition of 23 ± 0.2 months were used. The experiment started on d 21 AP and continued until d 105 PP. Before parturition, the heifers were kept in group pens and fed *ad libitum* with PMR (6.7 MJ NE_L/kg) comprising of 60% corn silage and 40% grass silage as well as 2 kg concentrate (6.7 MJ NE_L/kg) (Tab. A4; von Soosten et al., 2011). The heifers, which received the CTR (Silafat) or CLA (Lutrell Pure) after parturition, were housed in two group pens according to their diet. The PMR fed during the PP consisted of 25% grass silage, 38% corn silage and 37% PMR-concentrate on a DM basis. In addition, 4 kg concentrate containing the CTR fat or CLA supplements (100 g/d each) were fed (Tab. A5; von Soosten et al., 2011). The CLA

supplementation started on d 1 PP and continued until slaughter. On the day of parturition, 5 animals were slaughtered for determining the body composition and the weight of various organs as well as for collecting samples from SC and VC fat depots. The remaining 20 heifers were randomly allocated to either CTR fat supplement (100 g/d, n = 10) or CLA supplement (100 g/d, n = 10). Five animals each of the CTR and CLA group were then slaughtered on d 42 PP and on d 105 PP. The FA profiles for both of CLA and CTR fat supplements are shown in Tab. A3 (Pappritz et al., 2011; von Soosten et al., 2011). The CLA concentrate contained 1.7% of the t-10, c-12 and 1.6% of the c-9, t-11 CLA isomers (Tab. A5; von Soosten et al., 2011). The heifers of the CLA group consumed 6 g/d and 5.7 g/d of the t-10, c-12 and c-9, t-11 CLA isomers, respectively.

2.1.1.2.2. Sample collection

Samples of blood, milk and AT were collected for the CLA-PM-105 DIM experiment as demonstrated in Fig. 7. Blood samples were collected from the *vena jugularis externa* on d –21, –14, –7, –3, 1 (n = 25), 7, 14, 21, 28, 42 (n = 20) and 105 (n = 10) relative to parturition. The centrifugation of serum was performed after sampling, and the samples were stored at –80°C until analyzed (von Soosten et al., 2011). Milk samples were collected from each quarter of the 25 heifers on the respective slaughter days (d 1, 42 and 105) during the morning milking. After stunning and exsanguination, AT from 3 SC depots (tailhead, withers and sternum) and 3 VC depots (omental, mesenteric and retroperitoneal) were sampled and immediately fixed in 4% paraformaldehyde overnight. All AT depots were then dissected and weighted.

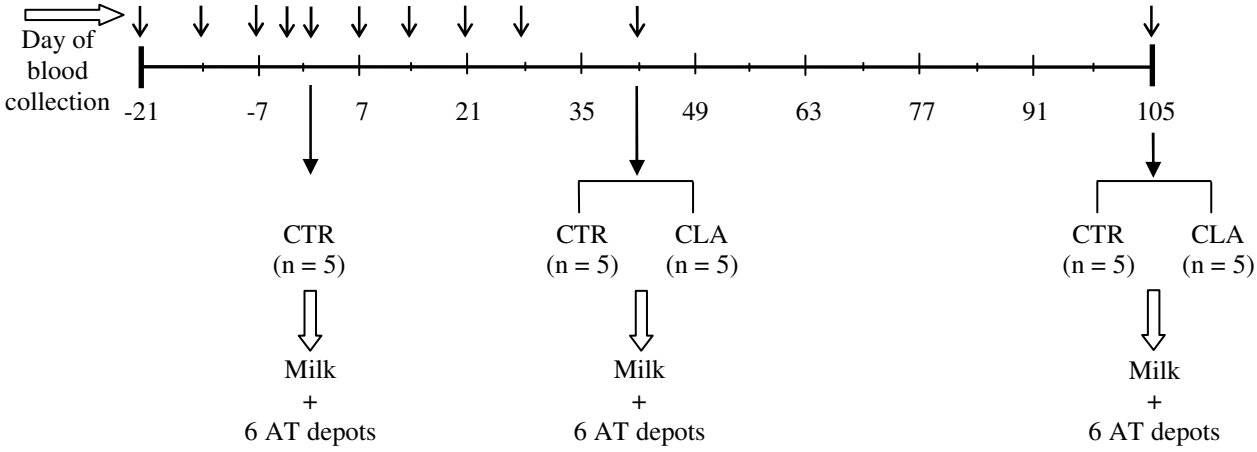

Fig. 7. Diagram of the experimental design and sample collection for the CLA-PM-105 DIM experiment. n: number of animals.

2.1.2. The concentrate-roughage ratio study

2.1.2.1. Treatments and diets

All animals were housed in a free stall barn at the experimental station of the Friedrich Loeffler Institute, Federal Research Institute for Animal Health, Braunschweig, Germany. The experimental design is described in detail by Locher et al. (2011). In brief, German Holstein dairy cows (n = 20) with a mean lactation number of 2.7 ± 0.18 were investigated from d 21 AP until d 21 PP. The cows were randomly allocated to two dietary treatments after calving. The first treatment was a low concentrate diet (**LC**, n = 10), in which the animals were fed as a total mixed ration (TMR) consisting of 30% concentrate (8.3 MJ NE_L/kg) and 70% roughage based on DM content. The second treatment was a high concentrate diet (**HC**, n = 10), in which the animals were fed a TMR consisting of 60% concentrate (8.2 MJ NE_L/kg) and 40% roughage. The feed ingredients and chemical composition of the PP diet are shown in Tab. A6 (Locher et al., 2011). During the experiment, no clinically relevant health problems occurred. Treatments were conducted according to the European Union regulations and were approved by LAVES, Germany (file number 33.14-42502-04-085/09).

2.1.2.2. Blood collection

Blood samples were harvested on d –21, –14, –7, –3, 1, 7, 14 and 21 relative to parturition. After centrifugation (10 min, 2,000 *g*), the serum was stored at –80°C until analyzed.

2.1.3. The fat heifers restriction study

2.1.3.1. Treatments and diets

The animal experiment was approved by the North Rhine-Westphalian State Agency for Nature, Environment and Consumer Protection (LANUV-NRW, file number 8.87.51.05.20.10.103, Recklinghausen, Germany). Twelve non-lactating, non-pregnant Simmental heifers with a mean BCS of 5 were housed in a tie stall barn at the research farm at Frankenforst, University of Bonn, Germany. The heifers were fed according to the recommendation of the German Society of Nutrition Physiology (GfE, 2001). The heifers (n = 12), with an initial live weight of 692 ± 43.1 kg, were allocated to two different treatments (Fig. 8). During the adaptation period, which lasted for three weeks, all heifers received grass

silage (9.5 MJ/kg DM) *ad libitum*. After the adaptation period, the heifers were either continuously fed with grass silage as before (100%; n = 6), or were restricted to grass silage diluted with straw (hay/silage: straw mixture (8.1 MJ/kg DM) (37:63); n = 6) for 4 weeks. Thereafter, all animals received again the grass silage for further 3 weeks. The chemical composition of the diet is shown in Tab. A7.

For synchronizing the heifers' estrous cycle, progesterone-releasing intravaginal device (PRID®alpha, Ceva Sante Animale, Liboure, France) were inserted 12 days before the onset of the experiment, and were replaced regularly every 3 weeks. In addition, 2 mL prostaglandin (Estrumate®, Intervet, Unterschleißheim, Germany) were injected in each animal at the first day of progesterone-releasing intravaginal device insertion. The heifers' weight was recorded at the beginning of the experiment and one day before or on each day of fat biopsy sampling as shown in Fig. 8.

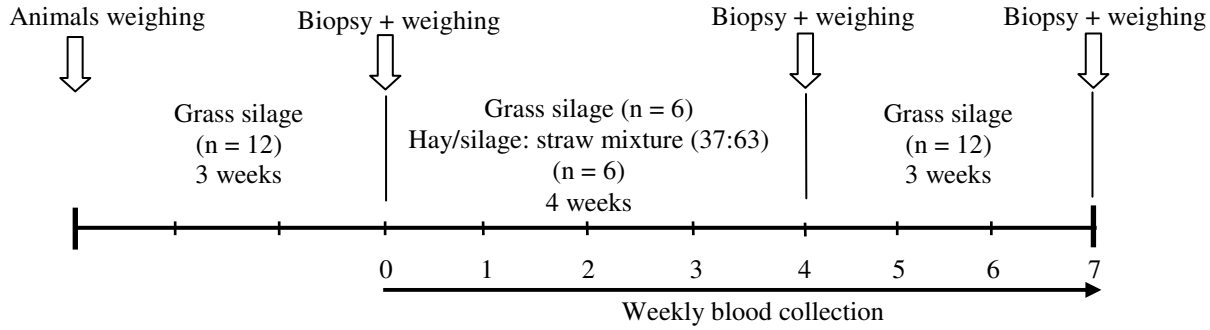

Fig. 8. Diagram of the experimental design and sample collection for the fat heifers restriction study. n: number of animals.

2.1.3.2. Sample collection

Blood samples were weekly collected from the coccygeal vein starting from the differential feeding period (Fig. 8). After centrifugation (20 min, 1,200 *g*, 4°C), the serum was stored at –20°C until analyzed. Biopsy samples from SC tailhead were collected from each heifer on the 0, 4th and 7th week (Fig. 8).

2.2. Laboratory analyses

All chemicals, buffers and solutions used in the laboratory analyses are listed in the appendix C.

2.2.1. Haptoglobin

Haptoglobin was detected by using the following methods: an enzyme-linked immunosorbent assay (ELISA), immunohistochemistry (IHC) and Western immunoblotting.

2.2.1.1. Measurement of haptoglobin in blood and milk by enzyme-linked immunosorbent assay

A competitive ELISA as described by Hiss et al. (2004) was used. The assay was modified by Hiss et al. (2009) using a standard serum for coating the plates and for the calibration curve instead of Hp purified from bovine serum. The serum which was used as standard had been calibrated against a standard obtained from the European Union Concerted Action on the standardization of animal APP (QLK5-CT-1999-0153; Skinner, 2001). The limit of detection was 0.07 µg/mL, and the intra- and interassay coefficients of variation (CV) were 3.8% and 5.6%, respectively. For performing the ELISA, 96 well-microtiter plates (EIA plate 9018, Corning Coster, Cambridge, MA) were coated with the calibrated bovine serum (dilution 1:3,000 in coating buffer) at 4°C for 20 h. After blocking with 250 µL of 2.5% casein solution at room temperature (RT) for 2 h, the plates were washed 5 times with washing buffer by using a microtiter plate auto washer (EL405; Bio-Tek Instruments, Inc., Winooski, VT, USA), and stored at –20°C prior to use. To start the test, 50 µL of the standard solution at serial dilutions of 0.012, 0.037, 0.11, 0.33, 1, 3 and 9 µg/mL were added in duplicate. The controls with known concentration of Hp were added to the plate in duplicate. The samples with 50 µL volume were added in duplicate. Fifty µL of the antiserum (polyclonal rabbit antiserum against bovine Hp (dilution 1:100,000 in test buffer)) were then added and incubated for 2 h on a shaker (M-1000; Med Tec, New Bern, NC, USA) at RT. After 3 washes, 100 µL of the second antibody (goat anti-rabbit IgG coupled with horseradish peroxidase (HRP) (Sigma-Aldrich Chemie, Taufkirchen, Germany, dilution 1:400,000 in test buffer)) were added and incubated for 30 min in a dark place. After 5 washes, the wells were filled with 140 µL of substrate solution and incubated in a dark place. The reaction was stopped after 30 min with

50 µL of 1 M oxalic acid, and the optical density (OD) was determined at 450 nm with a microplate reader (EL800; Bio-Tek Instruments). The concentrations of Hp in the samples were calculated from the calibration curve using the 4-parameter method of the Gen5™ data analysis software (Bio-Tek Instruments).

2.2.1.2. Detection of haptoglobin in adipose tissue by immunohistochemistry

The AT samples from the CLA-PM-105 DIM experiment were immediately fixed overnight in 4% paraformaldehyde (Roth, Karlsruhe, Germany, in phosphate buffered saline (PBS), pH 7.4), dehydrated in ascending grades of isopropanol (Roth)) (40%, 70%, 80%, 96%, and 2 times 100%), cleared in Roti®-Histol (Roth), and infiltered with a mixture of Roti®-Histol and melted paraffin (1:1, vol/vol) at 60°C. Finally, the samples were embedded in paraffin wax. The samples were cooled with Cryo-spray (Roth), sections (10 µm) were cut using a rotation microtome (SLEE, Mainz, Germany), mounted on SuperFrost®plus slides (Menzel, Braunschweig, Germany), dried at 60°C for 2 h, and hereafter at 37°C overnight. Sections were deparaffinized in Roti®-Histol, then gradually rehydrated in descending grades of isopropanol (100% (2 times), 96%, 80%, 70%, and 40%), and washed with distilled water. Antigen retrieval was performed with citrate buffer (0.01 mM pH 6) in a microwave oven for 3 x 5 min at 700 W, and then the sections were allowed to cool at RT for 30 min, and washed with distilled water. The elimination of endogenous peroxidase activity was done using 3% H_2O_2 for 15 min, the sections were washed 3 x 5 min with PBS (pH 7.2, 0.05% Tween®-20), and the unspecific binding was blocked with normal goat serum (1:10) for 20 min. Thereafter, the sections were incubated with a polyclonal rabbit antiserum against bovine Hp (Hiss et al., 2004; 1:2,000, in PBS) for 15 h. The sections were subsequently incubated with goat anti-rabbit IgG coupled with HRP (1:200 in PBS) for 30 min. The sections were then stained with 3-amino-9-ethylcarbazole (Biozol, Eching, Germany) as a substrate and counterstained in Mayer's Haematoxylin (Merck Eurolab GmbH, Darmstadt, Germany). After rinsing with tap water for 10 min, the sections were mounted with Kaiser's glycerol gelatin (Merck Eurolab GmbH). Based on previous findings that Hp mRNA is expressed in bovine leukocytes (Thielen et al., 2005), bovine lymph nodes were used as positive and negative controls (sections' thickness: 6 µm). For negative controls, the primary antibody was replaced with non-immune rabbit serum. The sections were evaluated through a Leica DMLB microscope (Leica Microsystems, Wetzlar, Germany) at 200x magnification and photographed with a digital camera (JVC, Hachioji Plant of Victor Company, Tokyo, Japan). Positive cells were

counted within the boundaries of 10 random microscopic fields per section using DISKUS software (Hilgers, Königswinter, Germany), and the percentage of positive cells was calculated as the number of positive cells/total number of cells x 100.

2.2.1.3. Detection of haptoglobin by Western immunoblotting

Homogenates from fat (n = 1) and liver (n = 1) tissue samples from the CLA-PM-105 DIM experiment were prepared by chopping the frozen sections into small pieces. Then, the tissues (0.6 to 0.7 g) were transferred to Precellys®-tubes pre-filled with 1.4 mm ceramic beads (peQLab Biotechnology, Erlangen, Germany). Thereafter, the tubes were filled with 900 and 600 µL of the homogenization buffer at pH 7.4 (10 mM HEPES, Applichem, Darmstadt, Germany) for the liver and fat tissue samples, respectively. The samples were homogenized 2 times for 15 sec using a Precellys®24 homogenizer (peQLab Biotechnology). Afterwards, a protease inhibitor (Complete™, Roche, Mannheim, Germany) was added (45 µL/900 µL homogenization buffer for liver and 30 µL/600 µL homogenization buffer for fat), the homogenates were then vortexed, and finally centrifuged (10 min, 14,000 *g*). The supernatant was re-centrifuged (10 min, 14,000 *g*, 4°C), and the resulting supernant was then frozen at –80°C.

The Western immunoblotting analysis of Hp was performed as described by Hiss et al. (2004). The homogenized SC tailhead and liver tissue (10 µg/lane) as well as one bovine serum sample (1:200 in H_2O) were each mixed with 1 µL dithiothreitol (5 M DTT, Applichem) and 10 µL sample buffer (5-fold, 20% sodium dodecyl sulfate (SDS)), boiled at 95.7°C for 8 min and centrifuged (5 min, 10,000 *g*). The biotinylated molecular weight marker (RPN 2107, GE Health Care, Amersham, Buckinghamshire, UK) (5 µg/lane) was used, and boiled at 97.5°C for 8 min. The marker and samples were loaded on 5.6% stacking gel and separated using 12% resolving gel of acrylamide (Roth). The SDS polyacrylamide gel electrophoresis (PAGE) was performed starting with 50 V for 30 min, and then followed by 150 V (Hoefer®pharmacia, Biotech, Inc., San Francisco, CA, USA).

Polyvinylidene fluoride (PVDF) membranes (Hybond™-P, GE Health Care) were activated with methanol (10 sec), washed with distilled water (5 min), and finally embedded with blotting buffer II (10 min). Ten filter papers were used, in which 3 of them were incubated with blotting buffer I (pH 10.4, 0.3 M Tris/HCl, 10% methanol), 2 papers with blotting buffer II (pH 10.4, 25 M Tris/HCl, 10% methanol), and the other 5 papers with blotting buffer III

(pH 9.4, 25 M Tris/HCl, 10% methanol). The resolving gel was equilibrated with blotting buffer II (10 min). Then the filters, membranes and the resolving gel were all sequentially placed in a semi-dry Electro-Blotter (STARLAB, Ahrensburg, Germany). The proteins were electrically transferred from the gel to the membrane for 30 min (30 mA).

Thereafter, the membranes were blocked with Roti®block (Roth) (diluted 1:10 in TBST (Tris-Buffered Saline, 0.05% Tween®-20)) on the shaker (10 rpm) for 1 h. The membranes were incubated with the polyclonal rabbit antiserum against bovine Hp (diluted 1:240,000 in Roti®block with TBST) on the shaker (10 rpm) for 1 h. Then, the membranes were washed 4 x 5 min with TBST, and incubated with the goat anti-rabbit IgG coupled with HRP (diluted 1:250,000 in Roti®block with TBST) on the shaker (10 rpm) for 1 h. The incubation with streptavidin-peroxidase (diluted 1:700,000 in Roti®block with TBST) was performed on the shaker (10 rpm) for 1 h after washing 4 x 5 min with TBST. The membranes were washed 4 x 5 min with TBST, placed in a plastic bag, and incubated with a mixture of equal volumes of each the enhanced chemiluminescence detection kit solutions A and B (ECL; RPN 2135, GE Health Care) for 5 min in darkness. The surplus from the detection kit was removed, and the membranes were placed under a CL-XPosure™ Film (Thermo Scientific). Finally, the film was exposed for 2 to 5 min and developed immediately. The obtained bands were visually evaluated according to their molecular weight without a densitometric analysis due to the low number of samples (n = 2).

2.2.2. Measurement of leptin in blood by enzyme-linked immunosorbent assay

Analysis of serum leptin was performed using a competitive ELISA as published by Sauerwein et al. (2004). The limit of detection was 0.3 ng/ml, and the intra- and interassay CV were 3.8% and 9.3%, respectively. The microtiter plates were coated by incubating 150 ng sheep 6 anti-rabbit-Fc fragment in the coating buffer with a volume of 100 µL/well at 4ºC for 20 h. After blocking the free binding sites with 200 µL of 2.5% casein solution at RT for 2 h, the plates were washed 5 times, filled with 200 µL test buffer/well, and stored at 4ºC. For performing the assay, the plates were decanted and 50 µL of the antiserum (polyclonal rabbit anti-leptin, diluted 1:12,000 in test buffer) with test buffer containing 1% goose serum were added. The controls with known concentrations of leptin and the serial standard dilutions (Recombinant ovine leptin, 0.11, 0.33, 1, 3, 9 and 27 ng/mL) were each pipetted (50 µL) in duplicate into the wells. Fifty µL of serum samples (diluted 1:2.5 in test buffer) were added

into each well. After 5 min shaking (M-1000; Med Tec), the plates were incubated for 16 h at 25ºC using a Thermostatic cabinet (Aqualytic®GmbH, Dortmund, Germany), where all further incubation steps were done. After the incubation, 50 µL of biotinylated recombinant leptin (Tracer, diluted 1:3,000 in test buffer) were added. After 2 h shaking with a shaker (MTS 4; IKA®SCHUTTER WERKE, Phoenix, AZ, USA, 250 rpm), the plates were decanted and washed 3 times, filled with 100 µL streptavidin-peroxidase (Southern Biotech, diluted 1:15,000 in test buffer) and incubated for 45 min. After washing 5 times, the last incubation step was performed using 140 µL of substrate solution and incubated for 45 min. Then the reaction was stopped with 50 µL of 1 M oxalic acid. The OD was measured at 450 nm, and leptin concentrations were calculated as previously mentioned for the Hp ELISA.

2.3. Statistical analyses

All statistical analyses were performed using SPSS software, version 17.0 (SPSS, Inc., Chicago, IL, USA).

For the CLA-252 DIM experiment, the mixed model procedure was used to analyze the data of serum Hp and leptin. Treatment (CTR, CLA-50 and CLA-100) was considered as a fixed factor, the sampling date (time) as a repeated effect and the respective interaction (time x treatment) was included into the model. The parity (cows vs. heifers) was considered as a fixed effect together with its interaction with the treatment. Parturition was considered via nested periods (before and after parturition) within time. For Hp, the clinical observations (diseased or not diseased) were considered in the model as a random effect for taking disease specific variations among groups into account. The covariance structure heterogeneous first-order autoregressive initially tested, and the one with the lowest value of the Akaike's information criterion was then applied. Comparisons among means were done using the Bonferroni test ($P \leq 0.05$).

For the CLA-PM-105 DIM experiment, where the repeated design was not applicable, all data were first tested for homogeneity of variances. In case of inhomogeneous variances, the non-parametric Mann-Whitney test was used to compare the CTR vs. CLA group. Disease as a random effect for Hp was tested using the general linear model (GLM). For IHC data, the GLM was used to perform the comparisons within the CTR and the AT depots of both CTR and CLA treatments at $P \leq 0.05$. To compare between the CTR and CLA treatments as well as between SC and VC depots, the student t-test was used and significant differences were

determined at $P \leq 0.017$ after Bonferroni α-correction for multiple comparisons (n = 3 comparisons; $\alpha = 0.05/3 = 0.017$).

For the "Fat heifers restriction study", the mixed model was used, in which Hp or leptin was considered as a dependent variable; group (animals fed grass silage vs. animals fed silage: straw mixture) as a fixed factor, time as a repeated effect, in addition, the respective interaction was included into the model.

For discriminating healthy from diseased animals by their Hp concentrations, the determination of the cut-off value was carried out using Youden's index (Youden, 1950) a function of sensitivity and specificity (sensitivity + specificity - 1). The Receiver Operating Characteristics' (ROC) curves were used to evaluate the quality of classification, in which the corresponding values for sensitivity and 1-specifity of various values were calculated and plotted against each other (Perkins and Schisterman, 2006).

Correlations were calculated using Pearson's correlation coefficient. The following general categories were used to describe the strength of correlation: $0 < r < 0.2$ (very weak), $0.2 \leq r < 0.4$ (weak), $0.4 \leq r < 0.6$ (moderate), $0.6 \leq r < 0.8$ (strong), and $0.8 \leq r < 1.0$ (very strong). All data are presented herein as means ± SEM.

3. Results

3.1. Conjugated linoleic acid studies

3.1.1. Haptoglobin

3.1.1.1. Serum haptoglobin

The timely changes of the concentrations of Hp during the entire experimental period (d –21 until d 252 relative to parturition) for both heifers and cows in the **CLA-252 DIM** experiment are shown in Fig. 9.

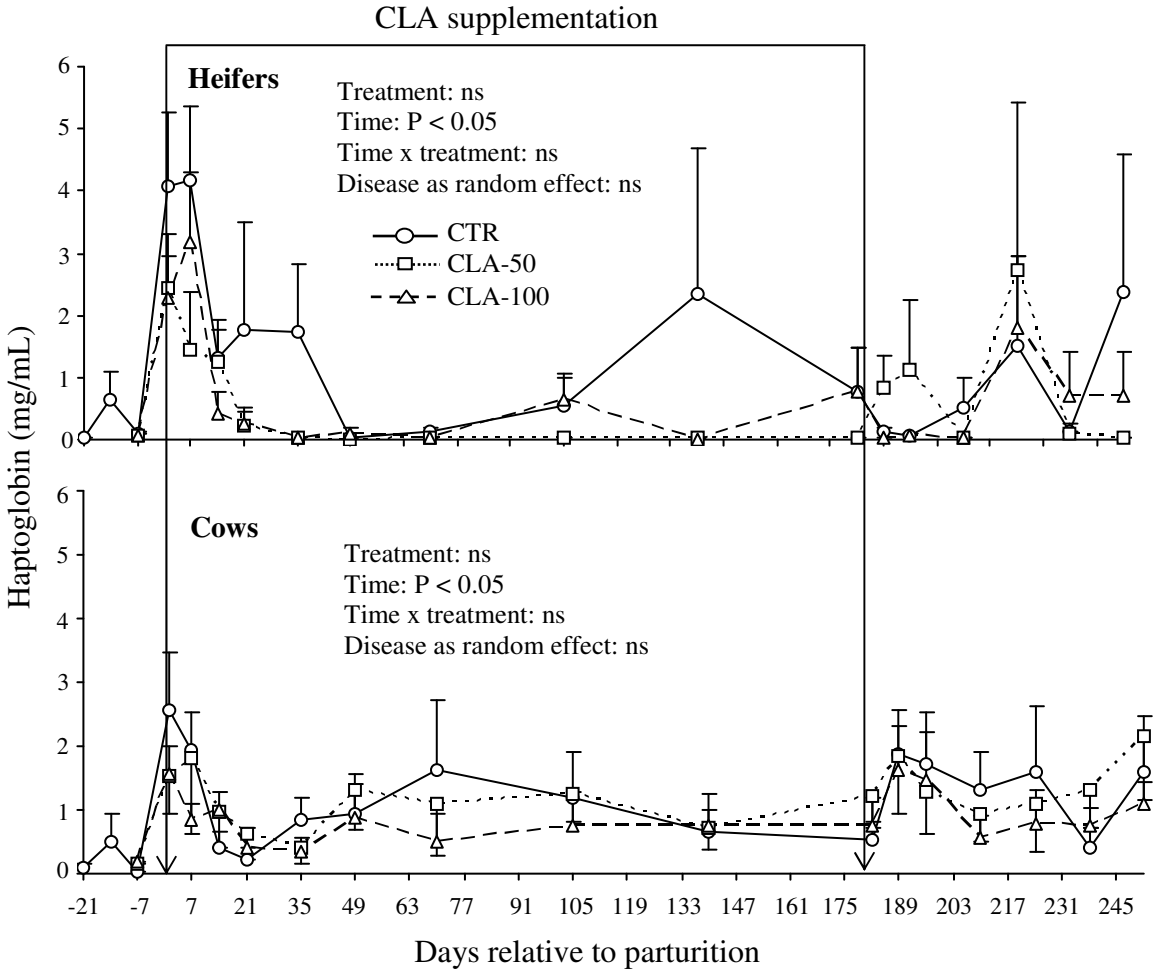

Fig. 9. Haptoglobin serum concentrations (means ± SEM) in heifers and cows receiving conjugated linoleic acid (CLA, Lutrell Pure, BASF SE) at 50 or 100 g/day or a control fat supplement (CTR, Sila Fat, BASF SE) from d 1 until d 182 post partum in the CLA-252 DIM experiment. The statistical results included in the graph comprise the treatment and post-treatment period (d 1 - 252). [CTR: cows n = 11, heifers n = 6; CLA-50: cows n = 11, heifers n = 5; CLA-100: cows n = 11, heifers n = 5)]. ns: not significant.

The results of all animals examined in the entire period showed that the concentrations of Hp were affected by parity (heifers vs. cows; $P < 0.05$) and parturition (before vs. after; $P < 0.001$). The concentrations of Hp increased during the first week PP compared with the preceding values to 2.94 ± 0.98 mg/mL for heifers and 1.70 ± 0.67 mg/mL for cows. During the first week PP, the concentrations of Hp were higher in heifers than in cows. Hereafter, the concentrations of Hp were again decreased and irregularly fluctuated over the entire period for both parity groups. The statistical analysis over the PP period (d 1 - 252) for heifers and cows showed that only time was significant, whereas neither CLA treatment nor disease were significantly associated with the concentrations of Hp (Fig. 9). During the treatment period (d 1 - 182 PP), there were no significant differences in the concentrations of Hp among the different treatments (CTR, CLA-50 and CLA-100) in both heifers and cows.

In the **CLA-PM-105 DIM** experiment, the timely changes of the concentrations of Hp in heifers throughout the entire period (d –21 until d 105 relative to parturition) are presented in Fig. 10.

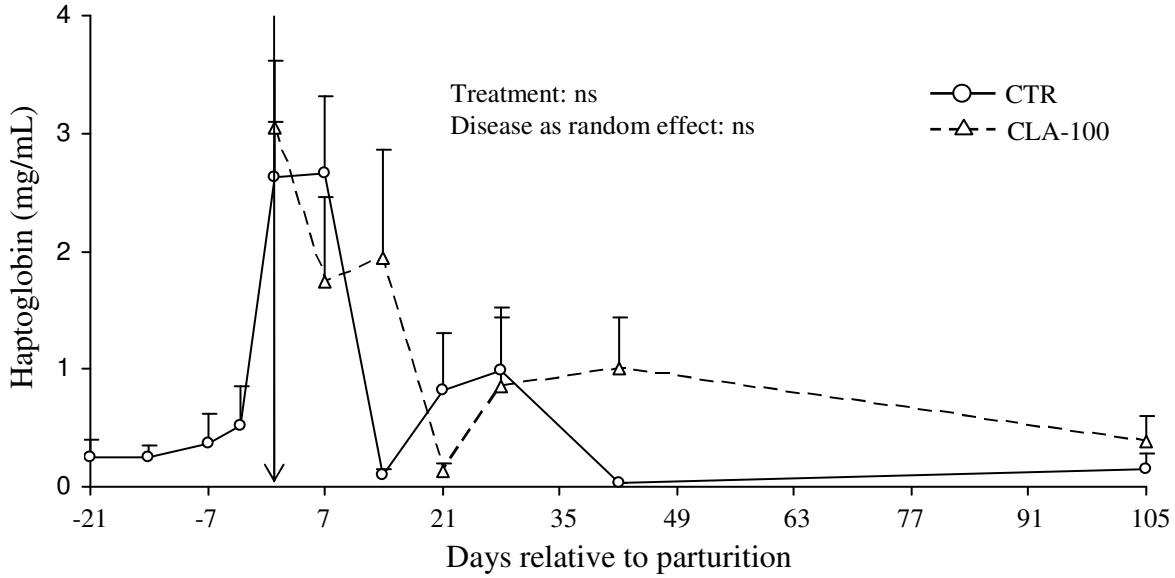

Fig. 10. Haptoglobin serum concentrations (means ± SEM) in heifers receiving conjugated linoleic acid (CLA, Lutrell Pure, BASF SE) at 100 g/day or a control fat supplement (CTR, Sila Fat, BASF SE) from d 1 until d 105 post partum in the CLA-PM-105 DIM experiment. The day of parturition is indicated by a vertical line. The statistical results included in the graph comprise the treatment period (d 1 - 105). [d –21 - 1: n = 25; d 7 - 42: n = 20 (10 each in CLA and CTR); d 105: n = 10 (5 each in CLA and CTR)]. ns: not significant.

The concentrations of Hp were affected by parturition ($P < 0.001$) with low values 3 weeks AP (0.35 ± 0.21 mg/mL), and elevated values during the first week PP (2.52 ± 0.61 mg/mL). Hereafter, the concentrations of Hp decreased again until d 105 PP. Further statistical analysis of the results revealed no significant differences in the concentrations of Hp during the first week PP between the heifers in this experiment and those in the CLA-252 DIM experiment. There were no differences in the concentrations of Hp between the CLA and CTR animals, as well as the concentrations of Hp were not affected by the health status of the animals (Fig. 10).

3.1.1.1.1. Relation of serum haptoglobin concentrations and body condition score, body weight, adipocyte size, fat mass, body fat content, concentrations of nonesterified fatty acids and β-hydroxybutyrate

The correlations were calculated for different periods relative to parturition in both experiments, and presented herein as the coefficients of significant ($P \leq 0.05$) correlations (summarized in appendix B; Tab. B1). Data for BCS, BW, adipocyte size, fat mass, NEFA and BHB were made available through the work of Akter et al. (2011), Pappritz et al. (2011) and von Soosten et al. (2011).

When testing the potential relationships between the concentrations of Hp and BCS or BW, no correlations were observed in the CLA-252 DIM and the CLA-PM-105 DIM experiments during the entire experimental period or at certain dates. Adipocyte size, fat mass and body fat content recorded in the CLA-PM-105 DIM experiment were also not correlated with circulating Hp concentrations.

For the cows in the CLA-252 DIM experiment, a very weak positive correlation was observed between the concentrations of Hp and NEFA in the following periods: d 21 AP - d 252 PP ($r = 0.14$, $P \leq 0.001$), d 1 - 252 PP ($r = 0.10$, $P \leq 0.05$), d 1 - 182 PP ($r = 0.12$, $P \leq 0.05$), and a weak positive correlation on d 189 - 252 PP ($r = 0.26$, $P \leq 0.001$). Concerning the BHB, a very weak positive correlation was recorded on d 21 AP - d 252 PP ($r = 0.12$, $P \leq 0.001$), d 1 - 252 PP ($r = 0.11$, $P \leq 0.05$), and d 1 - 182 PP ($r = 0.17$, $P \leq 0.01$).

For the heifers in the CLA-252 DIM experiment, a weak positive correlation was recorded between the concentrations of Hp and NEFA on d 21 AP - d 252 PP ($r = 0.38$, $P \leq 0.001$), a moderate correlation on d 1 - 252 PP ($r = 0.43$, $P \leq 0.001$), and d 1 - 182 PP ($r = 0.56$, $P \leq 0.001$). In contrast, no relation was established between the concentrations of BHB and Hp. In the CLA-PM-105 DIM experiment, a moderate positive relation between the concentration of

Hp and NEFA was reported during the entire experimental period (d 21 AP - 105 PP: r = 0.54, P ≤ 0.001), the AP period (d 21 - 7: r = 0.46, P ≤ 0.001), and the PP period (d 1 - 105: r = 0.49, P ≤ 0.001), while a weak positive relation was reported between the concentrations of Hp and BHB (d 1 - 105: r = 0.23, P ≤ 0.01 and d 21 AP - 105 PP: r = 0.20, P ≤ 0.01).

3.1.1.1.2. Haptoglobin as a measure of disease occurrence

Disease events and incidence rates

The disease events which were recorded for the animals in the CLA-252 DIM and the CLA-PM-105 DIM experiments are presented in Tab. 1 and 2, respectively. In total, there were 59 disease events in 41 animals (8 animals without any disease records) in the CLA-252 DIM experiment (Tab. 1), whereby more than one event at the same time and repeated events in one given animal had occurred. In the CLA-PM-105 DIM experiment, there were 32 disease events in 14 animals (11 animals without any records) (Tab. 2).

Tab. 1. Disease events recorded for animals (heifers and cows) in the control and the conjugated linoleic acid treatment groups during the CLA-252 DIM experiment.

Disease	Cows		Heifers	
	CTR (n = 11)	CLA (n = 22)	CTR (n = 6)	CLA (n = 10)
Mastitis	6	14	3	3
Metritis	3	8	4	4
Retained placenta	1	2	0	1
Mouth disease*	2	5	1	2

*Mouth disease = whitlow or phlegmon. n: total number of animals.

Tab. 2. Disease events recorded for heifers in the control and the conjugated linoleic acid treatment group during the CLA-PM-105 DIM experiment.

	d 1 (n = 5)	d 42 (n = 10)		d 105 (n = 10)	
Disease	CTR	CTR	CLA	CTR	CLA
Mastitis	1	0	2	3	3
Metritis	0	2	4	1	3
Retained placenta	1	1	2	1	1
Diagnoses on slaughter day*	1	1	1	1	3

*Abnormal findings during the slaughter process such as lung, liver, udder, uterus and rumen abscess. n: total number of animals.

The incidence rates ([number of cases/(number of animals x observation period (252 or 105 d))] x 100) for the animals in the CLA studies (CLA-252 DIM and CLA-PM-105 DIM) are presented in Tab. 3, in which the rates were calculated regardless of the treatment. For mastitis, the incidence rates were 0.24% (cows; equivalent to 0.24 cases/100 cow/d) and 0.15% (heifers; equivalent to 0.15 cases/100 cow/d) in the CLA-252 DIM experiment, and 0.34% (heifers; equivalent to 0.34 cases/100 cow/d) in the CLA-PM-105 DIM experiment. The incidence rates for metritis were 0.13% (cows; equivalent to 0.13 cases/100 cow/d) and 0.20% (heifers; 0.20 cases/100 cow/d) in the CLA-252 DIM experiment, and 0.38% (heifers; equivalent to 0.38 cases/100 cow/d) in the CLA-PM-105 DIM experiment. For retained placenta**,** the incidence rates were 0.04% (cows; equivalent to 0.04 cases/100 calvings) and 0.03% (heifers; equivalent to 0.03 cases/100 calvings) in the CLA-252 DIM experiment, and 0.23% (heifers; equivalent to 0.23 cases/100 calvings) in the CLA-PM-105 DIM experiment.

Tab. 3. Disease incidence rates* (%) in the CLA-252 DIM and the CLA-PM-105 DIM experiments.

	CLA-252 DIM		CLA-PM-105 DIM
Disease	Cows	Heifers	Heifers
Mastitis	0.24	0.15	0.45
Metritis	0.13	0.20	0.45
Retained placenta	0.04	0.03	0.29

* Disease incidence rate = [number of cases/(number of animals x observation period in days)] x 100.

Observation period = 252 and 105 d in case of the CLA-252 DIM and the CLA-PM-105 DIM experiments, respectively.

Example: In the CLA-252 DIM, 20 mastitis cases (6 CTR + 14 CLA) in cows.

Incidence rate = [20 mastitis cases/(33 x 252)] x100 = 0.24.

Determination of haptoglobin cut-off value

An optimal cut-off value of 0.16 mg/mL for the serum concentrations of Hp in the CLA-252 DIM experiment was determined using Youden index (Fig. 11) at maximal values of 59% sensitivity, 73% specificity and 0.32 for Youden index (Youden index (J) = max optimal cut-value (c) [sensitivity + specificity - 1]).

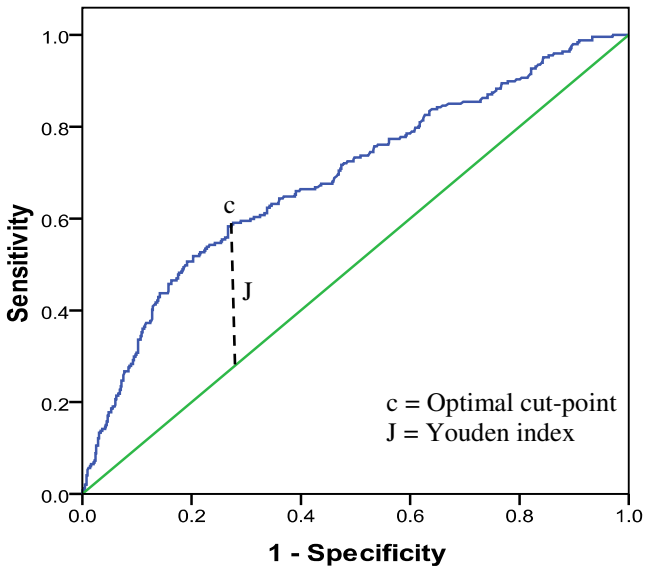

Fig. 11. ROC curve obtained using the concentrations of serum haptoglobin in the CLA-252 DIM experiment to differentiate between animals diagnosed with diseases and those without. The vertical line identifies the Youden index (J, dotted line), and the corresponding optimal cut-value (c).

Sample classification according to cut-off value of 0.16 mg/mL

The sample classification according to the cut-off value of 0.16 mg/mL in both CLA experiments in the PP period is presented in Tab. 4. In the CLA-252 DIM experiment, ˜ 60% of the serum samples of all animals in the CTR and CLA treatments had lower concentrations of Hp than 0.16 mg/mL, while ˜ 40% of the samples were higher than 0.16 mg/mL. In the CLA-PM-105 DIM experiment, 53% and 47% of the CTR and CLA samples had lower and higher concentrations of Hp than 0.16 mg/mL, respectively.

Tab. 4. Sample classification according to a Hp-cut-off value of 0.16 mg/mL serum in the conjugated linoleic acid studies.

Period[1]	Treatment	n[2] < Hp cut-off > n	Pearson chi-square (P < 0.05)
CLA-252 DIM	CTR	246 < 0.16 > 178	ns[3]
d 1 - 252	CLA	193 < 0.16 > 119	
CLA-PM-105 DIM	CTR	35 < 0.16 > 31	ns
d 1 - 105	CLA	35 < 0.16 > 30	

[1]Period relative to parturition.[2] number of samples below or above the cut-off value.[3]not significant.

Haptoglobin serum concentrations in healthy cows

The concentrations of serum Hp from d 21 AP until d 21 PP for cows in the concentrate-roughage ratio study are shown in Fig. 12.

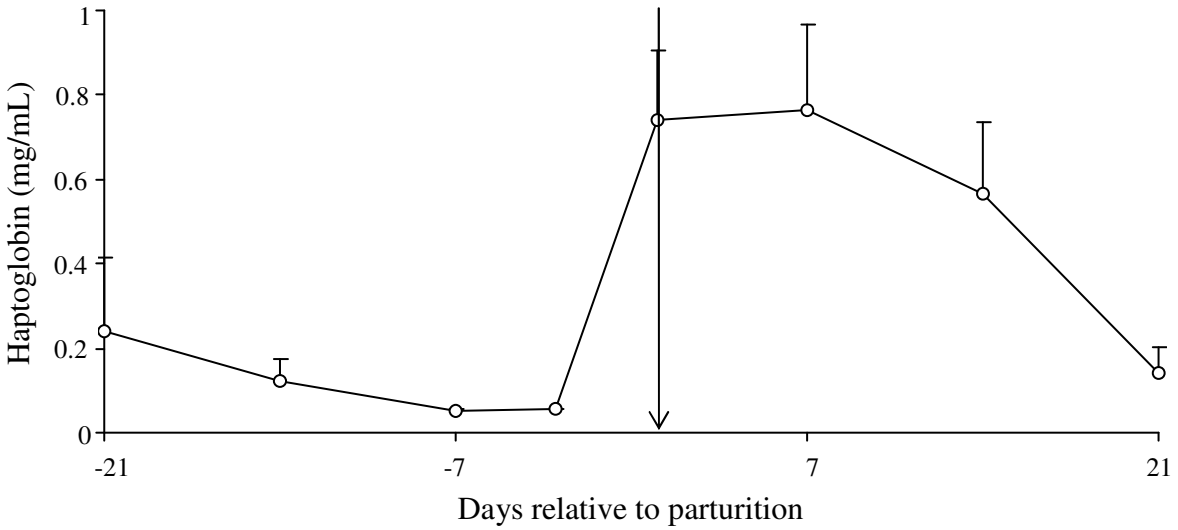

Fig. 12. Haptoglobin serum concentrations (means ± SEM) in cows (n = 20) observed from d –21 until d 21 relative to parturition in the concentrate-roughage ratio study. The day of parturition is indicated by a vertical line.

In the concentrate-roughage ratio study, where the cows fed LC (30% concentrate: 70% roughage) or HC (60% concentrate: 40% roughage) diets, the results indicated that the treatment had no significant effect on the concentrations of Hp, and therefore the data are presented herein as means from both treatments. The animals were apparently healthy; therefore, they were used to define the concentrations of Hp in healthy animals. The results showed that the concentrations of Hp peaked in the first week PP and then gradually declined until d 21 PP (Fig. 12). During the first week PP (as a mean from both d 1 and d 7), the cows used in this study had lower concentrations of Hp ($P < 0.05$) compared with cows (2.3-fold) and heifers (3.9-fold) used in the CLA-252 DIM experiment (Fig. 9), and heifers (2.4-fold) used in the CLA-PM-105 DIM experiment (Fig. 10).

3.1.1.2. Milk haptoglobin

Haptoglobin in the CLA-PM-105 DIM experiment was detected in 54 out of 96 milk udder quarter samples (56%), whereas 42 samples (44%) were below the limit of detection of 0.07 µg/mL.

Classification of the animals according to udder health status

The classification of the animals according to udder quarters into healthy (Hp < 2.2 µg/mL) and diseased (Hp ≥ 2.2 µg/mL) is presented in Tab. 5. The mean concentrations of milk Hp in the healthy quarters were 0.20 µg/mL (d 42 PP, CTR, n = 5), and 0.17 µg/mL (d 42 PP, CLA, n = 3). In contrast, the mean concentrations of milk Hp in the diseased quarters were 47.0 µg/mL (d 1 PP, CTR, n = 4), 53.6 µg/mL (d 42 PP, CLA, n = 2), 2.9 µg/mL (d 105 PP, CTR, n = 4), and 6.9 µg/mL (d 105 PP, CLA, n = 5).

Tab. 5. Classification of animals in the control and the conjugated linoleic acid treatment group according to a Hp-cut-off value of 2.2 µg/mL* milk in the CLA-PM-105 DIM experiment.

Classification	d 1 PP	d 42 PP		d 105 PP	
	CTR (n = 4)	CTR (n = 5)	CLA (n = 5)	CTR (n = 5)	CLA (n = 5)
Healthy quarters					
4/4 < 2.2 µg/mL	-	5	3	1	-
Diseased quarters					
1/4 ≥ 2.2 µg/mL	1	-	-	2	2
2/4 ≥ 2.2 µg/mL	1	-	-	1	1
3/4 ≥ 2.2 µg/mL	-	-	-	-	2
4/4 ≥ 2.2 µg/mL	2	-	2	1	-

*The cut-off value of 2.2 µg/mL was suggested by Hiss et al. (2007). n: number of animals.

The Hp milk concentrations in the healthy and diseased quarters regardless of the treatment are presented in Fig. 13. The mean concentrations of Hp were 0.15 and 21.4 µg/mL in the healthy and in the diseased quarters, respectively.

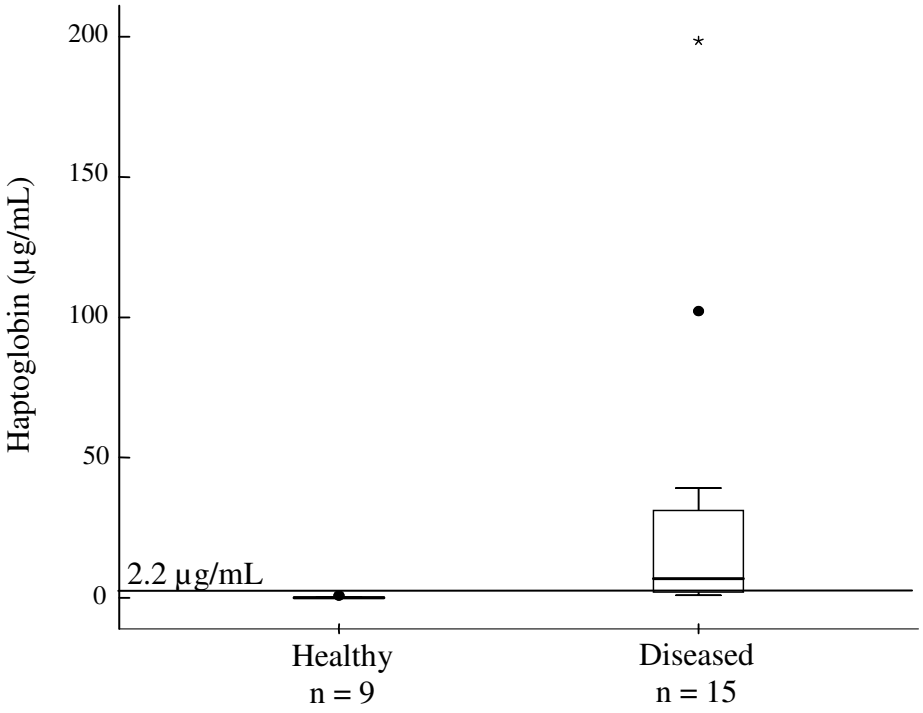

Fig. 13. Haptoglobin milk concentrations in healthy and diseased udder quarters in the CLA-PM-105 DIM experiment. The plots show the medians (line within box), 25th and 75th percentiles (box), 10th and 90th percentiles (whiskers). The horizontal line indicated the cut-off value of 2.2 µg/mL. n: number of animals. The dot and star stand for the outliers and extreme values.

The classification of the animals into healthy and diseased according to the SCC is summarized in Tab. 6.

Tab. 6. Classification of animals in the control and the conjugated linoleic acid treatment group according to the somatic cell count in the CLA-PM-105 DIM experiment.

Classification	d 1 PP	d 42 PP		d 105 PP	
	CTR (n = 2)	CTR (n = 2)	CLA (n = 3)	CTR (n = 5)	CLA (n = 5)
Healthy quarters					
4/4 < 200 x 10^3	-	2	-	1	-
Diseased quarters					
1/4 > 200 x 10^3	-	-	1	2	1
2/4 > 200 x 10^3	2	-	2	2	3
3/4 > 200 x 10^3	-	-	-	-	-
4/4 > 200 x 10^3	-	-	-	-	1

n: number of animals.

There were 26 out of 68 quarters (n = 17 heifers) with values > 200 x 10^3 cells/mL. The mean SCC values in the healthy quarters were 58 x 10^3 (d 42 PP, CTR) and 39.8 x 10^3/mL (d 105 PP, CTR), whereupon in the diseased quarters the mean values were 353 x 10^3 (d 1 PP, CTR), 1017 x 10^3 (d 42 PP, CLA), 340 x 10^3 (d 105 PP, CTR), and 463 x 10^3/mL (d 105 PP, CLA).

3.1.1.3. Presence of haptoglobin in adipose tissue

3.1.1.3.1. Qualitative description of haptoglobin by immunohistochemistry

Haptoglobin immune reactive staining was detectable in all tested SC and VC AT depots and in lymph node sections which were used as positive controls. In AT, Hp immunoreactivity was observed in the adipocytes, whereas the cells of the stromal-vascular fraction were all negative. In the adipocytes, Hp positive staining was evident along the cytoplasmic rim surrounding the large fat droplets (Fig. 14A), cytoplasmic rim edges (Fig. 14B) or as spot dots (Fig. 14C). Bovine lymph node tissue showed a punctuate, specific staining for Hp (Fig. 14D).

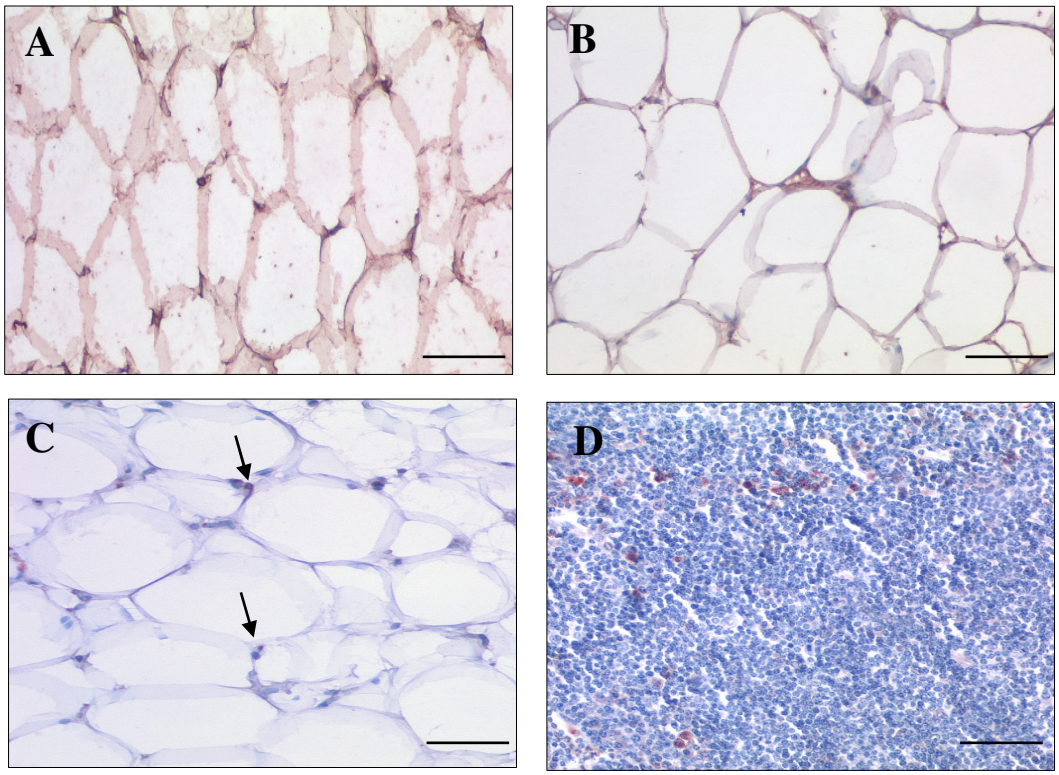

Fig. 14. Representative images showing haptoglobin positive staining in adipose tissue sections and in lymph node tissue used as positive control. (A) Along the cytoplasmic rim (retroperitoneal tissue); (B) Cytoplasmic rim edges (subcutaneous withers tissue); (C) Spot dots (subcutaneous withers tissue); (D) Positive control (bovine lymph node tissue). Original magnification: 200x. Bars represent 50 µm. Arrows indicated Hp positive staining (red color).

3.1.1.3.2. Detection of haptoglobin by Western immunoblotting

The primary antibody which was used in the immunohistochemical detection (as mentioned in the materials and methods section) was validated for it's reactivity in the liver and SC tailhead fat (Fig. 15A). The primary antibody reacted with bands of molecular weights from 20 to 34 kDa for liver and from 26 to 55 kDa for SC tailhead fat. The Western immunoblotting was used to detect any differences in the molecular weights between liver and serum samples as compared to SC tailhead fat (Fig. 15B). The results indicated that liver and serum bands were obtained on 34, 26 and 20 kDa molecular weights, while SC tailhead fat bands was obtained on 26 and 55 kDa molecular weights.

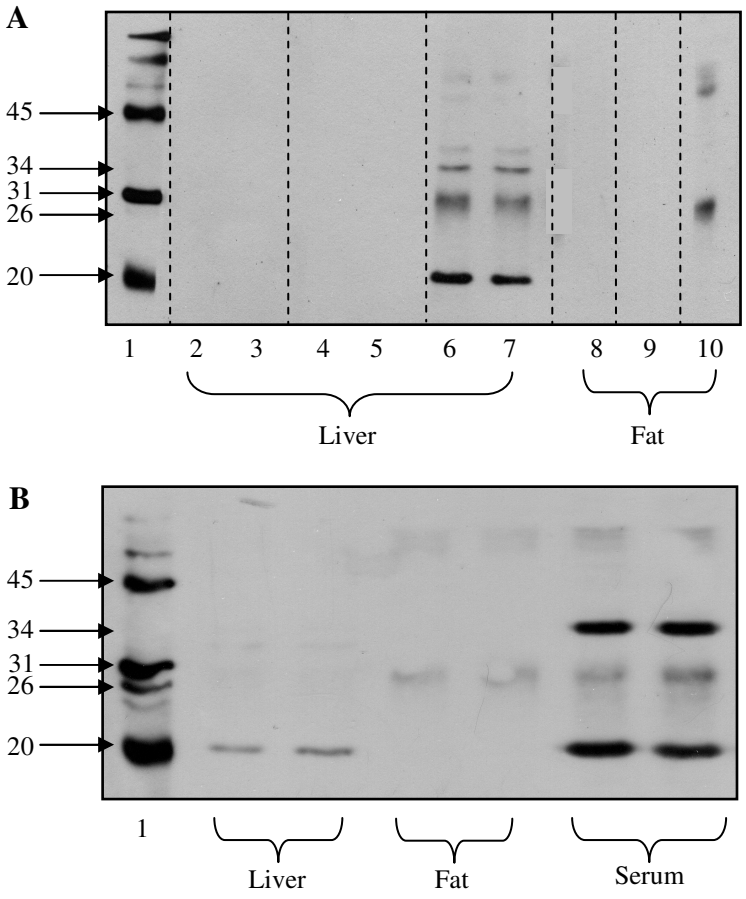

Fig. 15. Western immunoblot analysis of haptoglobin in bovine liver, subcutaneous tailhead fat and serum. (A) Test of antibody cross-reactivity in liver and subcutaneous tailhead fat. Lanes from left to right: (1) molecular marker in kDa; (2, 3, 8) - primary antibody and + secondary antibody; (4, 5, 9) + primary antibody and - secondary antibody; (6, 7, 10) + primary antibody and + secondary antibody. (B) Comparisons of liver, subcutaneous tailhead fat and serum.

3.1.1.3.3. Haptoglobin in subcutaneous and visceral adipose tissue

The results of histological localization of Hp indicated that VC fat had always higher portions of Hp positive cells than SC fat in both CLA and CTR treatments (Fig. 16).

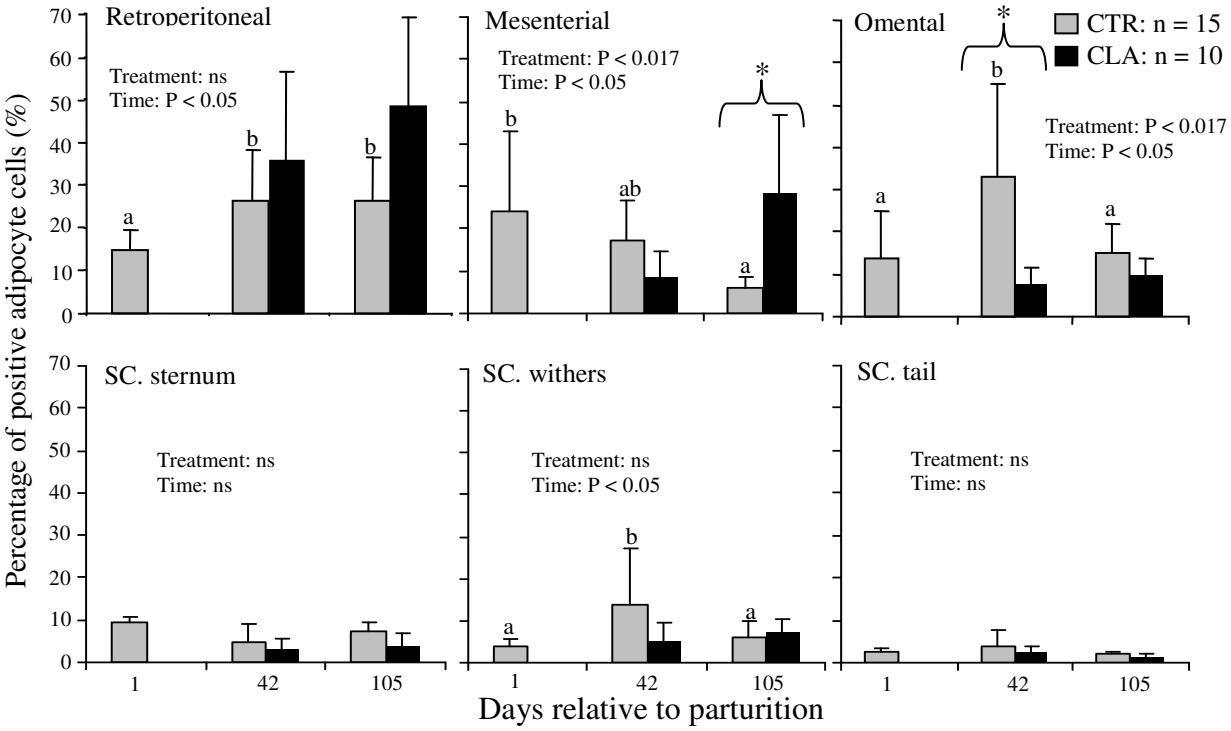

Fig. 16. Percentage (± SEM) of haptoglobin positive adipocyte cells in three visceral (VC) (upper panel) and three subcutaneous (SC) depots (lower panel) in heifers receiving conjugated linoleic acid (CLA, Lutrell Pure, BASF SE) or a control fat supplement (CTR, Sila Fat, BASF SE) at 100 g/day from d 1 until d 105 post partum in the CLA-PM-105 DIM experiment. Disease as random effect was not significant. Significant timely differences within the CTR group are shown by different letters; treatment effects are shown by brackets and stars. ns: not significant.

Comparing the different times of lactation, the VC depots underwent significant but inconsistent changes. In the CTR treatment, the percentage of Hp positive cells in the retroperitoneal tissue was significantly lower in d 1 PP compared to d 42 and 105 PP, in which the portion of percentage of positive cells increased 1.8-fold from d 1 to 105 PP. In contrast, mesenterial tissue from d 105 had only 25% of the values from d 1. For omental tissue, the portion of positive cells peaked on d 42, reaching 2-fold higher values than on d 1 and 105 PP. There were no significant differences in Hp positive cells in SC depots from sternum and tailhead with time, whereas the SC from withers had similar changes as observed in omental fat. Conjugated linoleic acid reduced significantly the percentage of Hp positive

cells in omental tissue by 4.3-fold (d 42) and increased significantly in mesenterial tissue in d 105 PP. No significant differences were found in the percentage of Hp positive cells between CTR and CLA treatments in the other tissues (Fig. 16).

3.1.2. Leptin

The concentrations of leptin for all animals (heifers and cows) from d –21 until d 252 relative to parturition in the CLA-252 DIM experiment are demonstrated in Fig. 17. There were no significant differences in the concentrations of leptin in terms of parity (heifers vs. cows), whereas the concentrations of leptin were affected by parturition (before vs. after parturition; $P < 0.001$). The concentrations of leptin were high in the AP period with a mean of 5.93 ± 0.53 ng/mL, and then the concentrations decreased PP below the pregnancy concentrations to 4.65 ± 0.43 ng/mL.

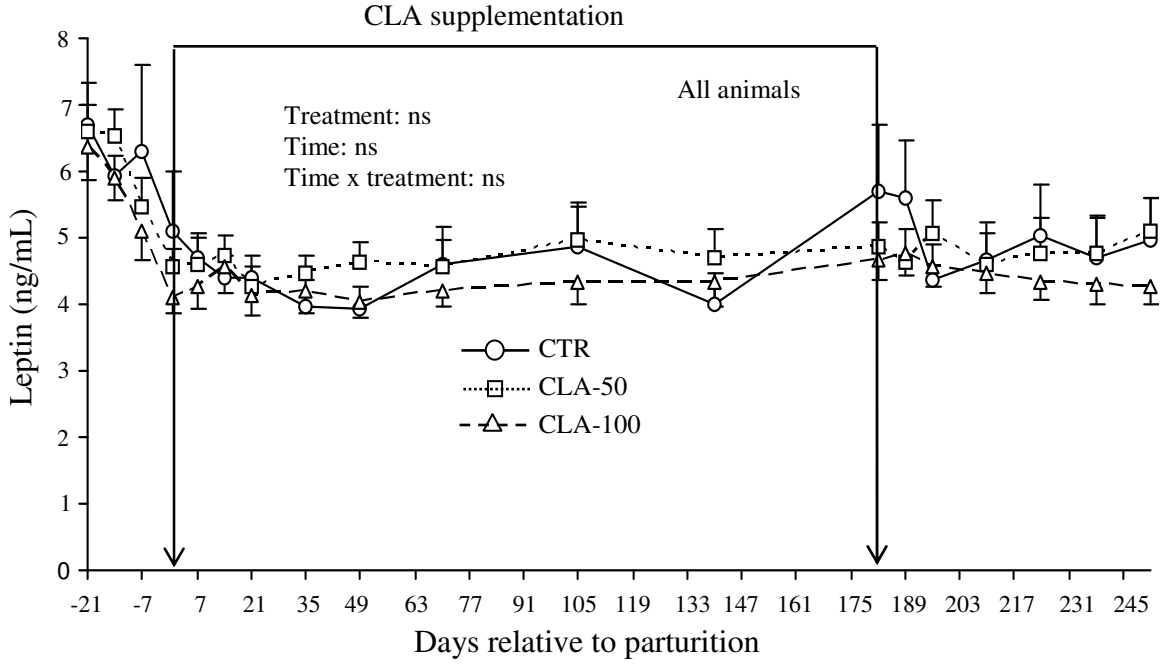

Fig. 17. Leptin serum concentrations (means ± SEM) in animals (n = 49) receiving conjugated linoleic acid (CLA, Lutrell Pure, BASF SE) at 50 or 100 g/day or a control fat supplement (CTR, Sila Fat, BASF SE) from d 1 until d 182 post partum in the CLA-252 DIM experiment. The statistical results included in the graph comprise the treatment and post-treatment period (d 1 - 252). [(CTR: n = 17; CLA-50: n = 16; CLA-100: n = 16)]. ns: not significant.

The statistical analysis from d 1 until d 252 PP for all animals confirmed no significant effects of CLA treatment, time and their interactions on the concentrations of leptin. During the treatment period (d 1 - 182 PP), the concentrations of leptin remained unaffected by CLA supplementation.

In the CLA-PM-105 DIM experiment, the changes in the concentrations of leptin from d –21 until d 105 relative to parturition are shown in Fig. 18. The concentrations of leptin were reduced ($P < 0.05$) PP (4.88 ± 0.46 ng/mL) as compared to AP (5.49 ± 0.54 ng/mL). The concentrations of leptin were not affected by CLA treatment (d 1 - 105 PP; Fig. 18).

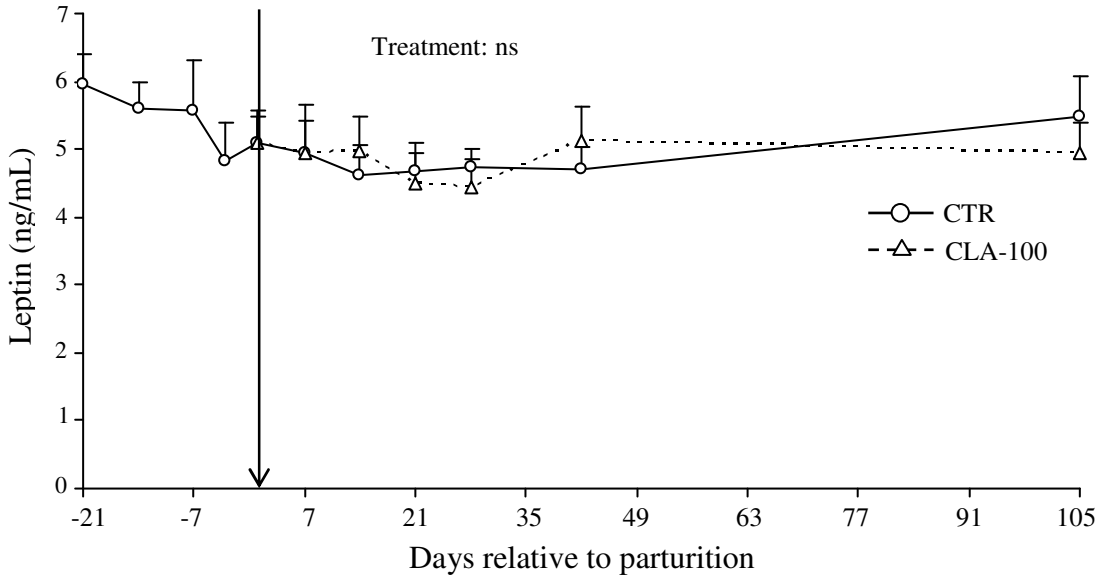

Fig. 18. Leptin serum concentrations (means ± SEM) in heifers receiving conjugated linoleic acid (CLA, Lutrell Pure, BASF SE) at 100 g/day or a control fat supplement (CTR, Sila Fat, BASF SE) from d 1 until d 105 post partum in the CLA-PM-105 DIM experiment. The day of parturition and CLA supplementation is indicated by a vertical line. The statistical results included in the graph comprise the treatment period (d 1 - 105). [(d –21 - 1: n = 25; d 7 - 42: n = 20 (10 each in CLA and CTR); d 105: n = 10 (5 each in CLA and CTR)]. ns: not significant.

Relation of serum leptin concentrations and body condition score, body weight, adipocyte size, fat mass, body fat content, concentrations of nonesterified fatty acids and β-hydroxybutyrate

The correlations were examined in different periods relative to parturition in both experiments, and presented herein as the coefficients of significant ($P \leq 0.05$) correlations (summarized in appendix B; Tab. B2 and B3). Data for BCS, BW, adipocyte size, fat mass, NEFA and BHB were made available through the work of Akter et al. (2011), Pappritz et al. (2011) and von Soosten et al. (2011).

In the CLA-252 DIM experiment, a very weak positive correlation between the concentrations of leptin and BCS was observed from d 21 AP until d 252 PP ($r = 0.19$, $P \leq 0.001$), and d 189 - 252 PP ($r = 0.18$, $P \leq 0.01$). In the CLA-PM-105 DIM experiment, a weak

positive relation between the concentrations of leptin and BCS was recorded in the AP period (d 21 - 7: r = 0.27, P ≤ 0.05) and in the PP period (d 1 - 105: r = 0.34, P ≤ 0.001). Concerning BW, a moderate positive relation (d 1 - 105: r = 0.46, P ≤ 0.001) was found only in the CLA-PM-105 DIM experiment.

In the CLA-PM-105 DIM experiment, there was a very strong positive correlation between the concentrations of leptin and adipocyte size on d 1 PP (mesenterial tissue: r = 0.98, P ≤ 0.01), and a strong positive relation on d 105 PP (retroperitoneal tissue: r = 0.75, P ≤ 0.05; SC withers: r = 0.78, P ≤ 0.01, and SC sternum: r = 0.71, P ≤ 0.05). A very strong positive correlation was recorded between leptin and fat mass on d 42 PP in omental (r = 0.90, P ≤ 0.001), retroperitoneal (r = 0.81, P ≤ 0.01), and SC (r = 0.83, P ≤ 0.01) as well as a strong relation in mesenterial (r = 0.71, P ≤ 0.05). A very strong positive correlation was recorded between the concentrations of leptin and body fat content on d 42 PP in omental (r = 0.86, P ≤ 0.001), retroperitoneal (r = 0.83, P ≤ 0.01), and SC (r = 0.80, P ≤ 0.01) as well as a strong relation in mesenterial fat (r = 0.72, P ≤ 0.05). A very strong positive correlation was found between the concentrations of leptin and the total fat mass (r = 0.88, P ≤ 0.001) and total body fat content (r = 0.91, P ≤ 0.001) on d 42 PP.

In the CLA-252 DIM experiment, a very weak inverse correlation was observed between the concentrations of leptin and NEFA in the following periods: d 21 AP - d 252 PP (r = -0.15, P ≤ 0.001), d 1 - 252 PP (r = -0.11, P ≤ 0.01) and d 1 - 182 PP (r = -0.1, P ≤ 0.05). In contrast, no correlation was reported between BHB and the concentrations of leptin in the CLA-252 DIM. In the CLA-PM-105 DIM experiment, no correlation was found between the concentrations of leptin and NEFA, while a weak positive relation was recorded with BHB (d 1 - 105: r = 0.34, P ≤ 0.001).

3.2. Effect of fat mobilization on the concentrations of haptoglobin and leptin in the fat heifers restriction study

The changes in the concentrations of Hp and leptin in the fat heifers restriction study are shown in Fig. 19A and B, respectively. There were no significant differences in the concentrations of Hp and leptin between animals fed either grass silage or hay/silage-straw mixture. In general, the concentrations of Hp and leptin varied slightly from week to week and did not show consistent changes across the investigated period.

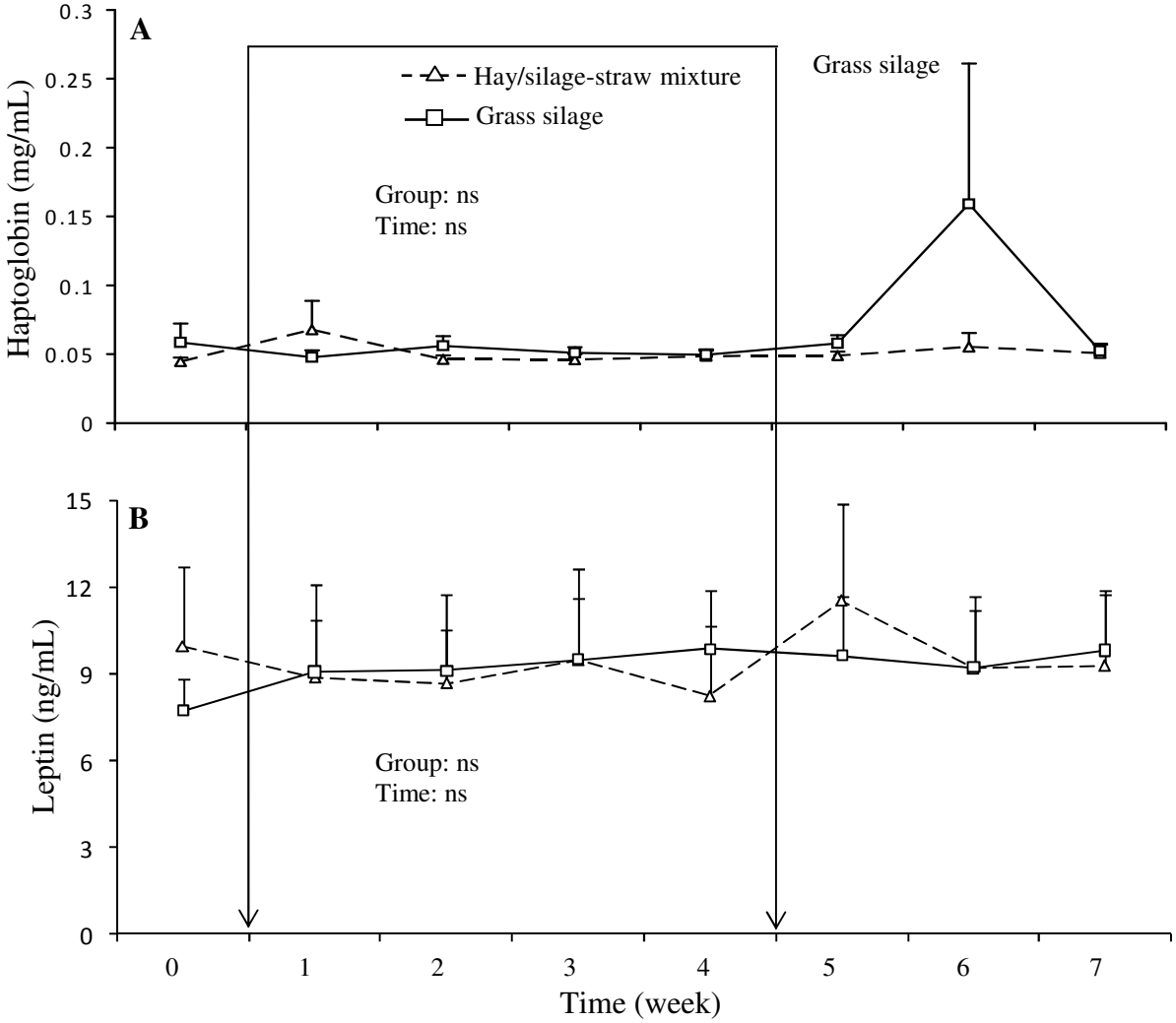

Fig. 19. Haptoglobin (A) and leptin (B) serum concentrations (means ± SEM) in non-lactating heifers receiving hay/silage-straw mixture (n = 6) or grass silage (n = 6) for 4 weeks and only grass silage (n = 12) for 3 weeks thereafter in the fat heifers restriction study. The vertical lines indicate the differential feeding period. The statistical results included in the graph comprise the differential feeding period. ns: not significant.

4. Discussion

4.1. Characterization of haptoglobin and leptin concentrations throughout lactation or during early lactation in the conjugated linoleic acid studies

The CLA studies consisted of two independent experiments; the CLA-252 DIM comprising heifers (n = 16) and cows (n = 33) observed from d 21 AP until d 252 PP, and the CLA-PM-105 DIM conducted on heifers (n = 25) only for a period from d 21 AP until d 105 PP.

Serum haptoglobin concentrations

The physiological changes observed during the CLA-252 DIM experiment were related to parity (heifers vs. cows) and parturition (before vs. after). During the first week after parturition, the concentrations of Hp were 1.7-fold higher in heifers than in cows. These results are in agreement with those obtained by Humblet et al. (2006) (study period: 8 weeks AP until 16 weeks PP), who demonstrated that heifers had 2.2-fold higher concentrations of Hp than cows in the first week following parturition. Moreover, Crawford et al. (2005) investigated heifers and cows from 3 weeks AP until 6 weeks PP and reported Hp concentrations being 1.3-fold higher in heifers than in cows during the first week after parturition. Furthermore, higher concentrations of Hp (1.9-fold) and ceruloplasmin (1.2-fold) have been reported during the first 27 d PP in heifers than in cows (Cullens, 2005). In contrast, Crawford et al. (2005) stated that there was no difference in the concentrations of Hp between cows and heifers in week 6 PP. The reason for the mostly higher concentrations of Hp recorded in heifers than in cows in the first week PP is not clear. It might be that parturition is more stressful for heifers than for cows. It could be concluded that our finding suggests a more intense physiological response to first calving than to later ones as suggested by Humblet et al. (2006).

In the current study, the concentrations of Hp peaked around calving and decreased afterwards in both the CLA-252 DIM and the CLA-PM-105-DIM experiments. This finding is in agreement with the studies of Uchida et al. (1993), Hachenberg et al. (2007), Tóthová et al. (2008) and Kováč et al. (2009). This increase is probably related to the hormonal changes and the tissue lesions occurring during birth (Young et al., 1995; Hachenberg et al., 2007). However, the increase in energy requirements during the transition period results in NEB, and

therefore energy has to be mobilized from AT. This process results in increased circulating concentrations of NEFA and formation of ketone bodies (Herdt, 2000). Thus, the transition period in dairy cows presents a risk for metabolic disturbances such as fatty liver and ketosis. The APR is associated with numerous changes in lipid metabolism such as accelerated lipolysis and increased NEFA in plasma (Hardardóttir et al., 1994). Hiss et al. (2009) and Kováč et al. (2009) suggested that in cows with higher concentrations of NEFA, higher concentrations of Hp might be found. However, Uchida et al. (1993) showed that experimentally induced fatty liver was associated with increasing concentrations of Hp; therefore a part of this increase in the first week PP could be caused by NEB and metabolic stress (Crawford et al., 2005). In the current study, the relation between the concentrations of serum Hp and each of NEFA and BHB (Pappritz et al., 2011; von Soosten et al., 2011) was assessed. In the CLA-252 DIM (cows and heifers) and CLA-PM-105 DIM (heifers) experiments, a positive relation was recorded between the concentrations of Hp and each of NEFA and BHB. This finding is in accordance with Kováč et al. (2009) who also found a positive correlation between Hp and some variables of energy metabolism such as NEFA and BHB. In both experiments (CLA-252 DIM and CLA-PM-105 DIM), the concentrations of Hp showed a stronger positive correlation with NEFA in heifers than in cows; therefore it might be that heifers are more stressed by lipolysis than cows.

The current study indicated that the time course of the concentrations of Hp after the first week of lactation did not show marked changes, which is in line with Humblet et al. (2006) (study period: 8 weeks AP until 16 weeks PP) who reported no significant effects of time on the concentrations of Hp after the first week PP.

Serum leptin concentrations

The present study demonstrated that physiological alterations in the concentrations of serum leptin in the CLA-252 DIM experiment were limited to parturition (before vs. after), whereas no parity (heifers vs. cows) differences could be found. Conversely, other researchers reported that the concentrations of leptin are affected by parity, whereby circulating leptin is significantly higher in heifers than in cows before calving (Wathes et al., 2007), and the peripartal decrease was steeper in heifers than in cows around calving (Meikle et al., 2004; Wathes et al., 2007). Heifers generally calve for the first time at about 24 months of age (Hoffman and Funk, 1992), and they are not physically mature at this stage (Coffey et al.,

2006). Thus, heifers approaching their first calving are in a differing metabolic state to that experienced by cows as they require nutrients for their own continued growth in addition to that of their developing calf (Wathes et al., 2007). However, the steeper decrease in the concentrations of leptin in heifers might be explained by that heifers showed a metabolic/endocrine profile less balanced (e.g. higher NEFA and BHB, lower insulin-like growth factor-I (IGF-I) and thyroid hormones) than cows (Meikle et al., 2004). This reflects that heifers are recovering from the NEB period with more difficulty. Ehrhardt et al. (2000) estimated that BCS in late pregnant cows explained 37% of the variation in plasma leptin. In addition, Wathes et al. (2007) reported that the higher circulating leptin in heifers before calving might relate to the tendency towards a slightly higher BCS than cows. Both heifers and cows used in the present study (CLA-252 DIM experiment) had a BCS of 3.5 before calving; therefore, this might explain why parity did not significantly affect the concentrations of leptin.

In the present study, the concentrations of leptin were high during late pregnancy and declined towards parturition, and then remained low in both CLA-252 DIM and CLA-PM-105-DIM experiments. This finding is in agreement with several previous studies (Block et al., 2001; Liefers et al., 2005; Hachenberg et al., 2007; Wathes et al., 2007). The high concentrations of leptin during pregnancy are due to an increase in body fat reserves and a concomitant increase in leptin mRNA expression in AT (Ehrhardt et al., 2001). The decline in the concentrations of plasma leptin towards parturition with the onset of NEB is probably caused by the inhibition of leptin expression by AT (Block et al., 2001), and increased mobilization of AT (Liefers et al., 2005). Recently published studies, using the same animals that were used in the current study, indicated that positive EB was achieved after d 49 (Pappritz et al., 2011) and d 14 PP (von Soosten et al., 2011) in the CLA-252 DIM and CLA-PM-105 DIM experiments, respectively. In addition, the highest mobilization of AT, according to the concentrations of NEFA, occurred from d 7 AP until d 21 PP in both experiments (Pappritz et al., 2011; von Soosten et al., 2011). The onset of NEB around parturition is associated with decreased plasma insulin and increased plasma GH (Block et al., 2001), suggesting that both hormones could mediate a portion of the effect of EB on plasma leptin and attribute to the reduction of leptin concentrations towards parturition.

In humans and rodents, leptin is synthesized in proportion to the overall degree of adiposity (Friedman and Halaas, 1998). Similar relationships have been observed between leptin and

body fatness in ruminants (Blache et al., 2000; Thomas et al., 2001; Altmann et al., 2006), BCS (Ehrhardt et al., 2000; Delavaud et al., 2002) and adipocyte size (Delavaud et al., 2002). In the current study, the relationship between the concentrations of serum leptin and each of BCS, BW (Pappritz et al., 2011; von Soosten et al., 2011), fat mass and body fat content (von Soosten et al., 2011), and adipocyte size (Akter et al., 2011) on different days relative to parturition was tested in both experiments. The concentrations of leptin had a stronger relation with depots fat mass, body fat content, and adipocyte size than BCS. These observations confirm those of Delavaud et al. (2002) who reported that plasma leptin is more strongly related to adipose cell size ($r = 0.91$) than to BCS ($r = 0.54$). This finding suggests that the increase in leptin that is observed when adiposity increases is strongly related to hypertrophy of adipose cells as reported by Delavaud et al. (2002).

In addition, the relationship between the concentrations of serum leptin and both of NEFA and BHB was tested in both experiments of the current study. It was found that the concentrations of leptin were negatively correlated with NEFA, and positively with BHB. The present results are in agreement with those of Block et al. (2001) and Accorsi et al. (2005) who reported that the concentrations of plasma leptin were negatively related to the concentrations of NEFA. Block et al. (2001) reported that the significant correlations between the plasma concentrations of leptin, insulin, GH, glucose and NEFA could represent co-regulation by EB, and perhaps a role for these factors in mediating the effect of EB on leptin synthesis. At the beginning of lactation, GH concentrations increased, while a reduction occurred in leptin and insulin. This endocrine condition, such as the significant increase in NEFA plasma concentrations, is indicative of a marked lipid mobilization. In the more advanced stages of lactation, when both energy and protein balances become positive, leptin plasma concentrations increased, whereas NEFA concentrations declined (Accorsi et al., 2005).

4.2. Effect of supplementation with conjugated linoleic acids on serum haptoglobin and leptin

Efficacy of the treatments with conjugated linoleic acids

The efficacy of the CLA supplementation in terms of milk fat reduction has been reported in both experiments using the same animals used in the present study (Pappritz et al., 2011; von Soosten et al., 2011). In the CLA-252 DIM experiment, Pappritz et al. (2011) reported that the CLA supplementation (d 56 until d 182 PP) resulted in a reduction of milk fat content by 7 and 12% in the CLA-50 and in the CLA-100 group, respectively. In the CLA-PM-105 DIM experiment, the CLA supplementation decreased milk fat content by 14.1% (d 1 until d 42 PP) and 25.4% (d 42 until d 105 PP) (von Soosten et al., 2011). In the CLA-252 DIM experiment, BW, BCS, plasma concentrations of glucose, BHB and NEFA did not differ among treatments over the CLA supplementation period although there was a trend for lower plasma NEFA concentrations in the CLA-100 animals from d 21 until d 182 PP (Pappritz et al., 2011). In the CLA-PM-105 DIM experiment, BW, BCS, NEFA, BHB and glucose were not changed by CLA supplementation in any period of experiment (von Soosten et al., 2011). Conjugated linoleic acids supplementation did not affect the weights of liver, VC (omental, mesenteric), or SC adipose depots with the exception of retroperitoneal fat, in which a trend to a lower weight of animals slaughtered on d 42 PP and d 105 PP was observed (von Soosten et al., 2011). Akter et al. (2011) reported that dietary CLA supplementation decreased adipocyte sizes of different SC and VC depots to different extents in dairy cows during the first 105 d PP. Moreover, the extent of CLA-induced decrease of adipocyte sizes is consistently higher at d 105 PP than at d 42 PP for both SC and VC depots. They concluded that CLA-induced decrease in adipocyte size indicates that CLA does affect body fat in dairy cows.

Effect of supplementation with conjugated linoleic acids on serum haptoglobin

The functioning of the immune system, like most systems in the body, is dependent on adequate nutrition. Moreover, nutrition signals can affect gene and protein expression which modulate the inflammation markers depending on the net changes in gene expression and resulting in both positive and negative effects (do Nascimento et al., 2009). Fatty acids are important modulators of the immune system (Calder, 2008; Serhan et al., 2008). In a number

of different animals, CLA has been suggested to have anti-inflammatory and/or immune-ameliorating effects (Yu et al., 2002). The c-9, t-11 and t-10, c-12 isomers of CLA induce a number of physiological effects, some by the independent actions of a single isomer, others by (synergistic) interactions involving both isomers (Pariza et al., 2001). Moreover, the c-9, t-11 and t-10, c-12 CLA isomers have been reported for possessing anti-inflammatory effects (Huebner et al., 2010). In contrast, the t-10, c-12 CLA isomer has also been reported to induce inflammation (Poirier et al., 2006). However, to the best of our knowledge this is the first report on the effect of CLA supplementation on Hp in ruminants. The present investigation demonstrated that long-term feeding of dairy cows with CLA containing 12% of the t-10, c-12 and c-9, t-11 CLA isomers in equal proportions, had no effect on the concentrations of serum Hp in both experiments. However, one study in rats indicated a reduction in the concentrations of serum Hp fed 1.5% (1.5 g/100 g of total ration) CLA isomers (t-10, c-12; c-9, t-11; c-11, t-13 and t-8, c-10) for 8 weeks (Noto et al., 2007). Contradictory results of other inflammation markers are reported in literature: supplementation with 3.4 g/d CLA (purified t-10, c-12) in men with metabolic syndrome for 12 weeks significantly increased CRP (2.2-fold), but no significant increase was found for TNF-α and IL-6 (Risérus et al., 2002). In non obese humans, 3.2 g/d CLA (equal amounts of c-9, t-11 and t-10, c-12 isomers) for 12 weeks increased CRP, but CLA supplementation did not have any significant effect on plasma concentrations of TNF-α (Smedman et al., 2005). In contrast, Moloney et al. (2004) found no effect of 3 g/d CLA (50:50 blend of c-9, t-11 and t-10, c-12) supplemented for 8 weeks, on CRP or IL-6 in human subjects with type 2 diabetes.

Effect of supplementation with conjugated linoleic acids on serum leptin

Leptin is a protein secreted from adipocytes that has been implicated in the regulation of food intake, energy expenditure and whole-body EB. However, because CLA is reported to reduce body fat content in humans (Thom et al., 2001), rats (Yamasaki et al., 2000), mice (DeLany et al., 1999), and pigs (Ostrowska et al., 1999) as well as to induce reductions in adipocyte size in rats (Poulos et al., 2001), mice (Tsuboyama-Kasaoka et al., 2000) and dairy cows (Akter et al., 2011), CLA supplementation has been linked to a reduction of circulating leptin concentrations in rats (Rahman et al., 2001), mice (Parra et al., 2010), and humans (Medina et al., 2000). In addition, CLA may act directly on leptin production because it could reduce leptin without affecting the BW and body fat content as reported in humans (Medina et al., 2000) and rats (Yamasaki et al., 2003). The t-10, c-12 CLA isomer has been suggested to

decrease and the c-9, t-11 CLA isomer to increase leptin secretion and gene expression (Rodríguez et al., 2002; Ahn et al., 2006; Gudbrandsen et al., 2009). However, the present results showed that long-term feeding of dairy cows with CLA containing 12% of the t-10, c-12 and c-9, t-11 CLA isomers in equal parts had no significant effect on the concentrations of serum leptin in both experiments. This finding is in line with a relatively short study (5 days) on dairy cows in mid to late lactation receiving 10 g/d abomasal infusions of either c-9, t-11 or t-10, c-12 CLA isomer and reporting no effect of CLA treatment on the concentrations of plasma leptin (Baumgard et al., 2002). In addition, Gillis et al. (2004b) reported that feeding beef heifers 2% (of total ration on DM basis) rumen-protected CLA salts (a mixture of Ca-salts of palm oil FA with 31% CLA (27.2% c-9, t-11; 32.8% t-10, c-12; 10.6% t-8, c-10; 18.95% c-11, t-13 and 10.5% various t, t CLA isomers)) on the last 32 or 60 days before slaughter had no effect on the concentrations of serum leptin.

Long-term CLA supplementation after calving left the concentrations of serum Hp and leptin unaffected. In this study, it is difficult to make definite conclusions on the long-term CLA supplementation effect on the concentrations of Hp and leptin. The reasons for the lack of a CLA effect are unknown, but it is likely that dose, duration and the isomeric composition of CLA separately affect the ability of CLA to influence the concentrations of Hp and leptin in dairy cows. In addition, the individual dietary intake from the c-9, t-11 and t-10, c-12 CLA isomers was lower than planned in the CLA-252 DIM and CLA-PM-105 DIM experiments. The aim of the CLA-252 DIM experiment was to achieve an individual dietary intake of 5 and 10 g/d of t-10, c-12 and c-9, t-11 CLA isomers in CLA-50 and CLA-100 groups, respectively. However, Pappritz et al. (2011) reported that the calculated intakes, based on the analyzed concentrations given in feed-concentrates, were 20% lower than expected (4 and 8 g/d of t-10, c-12 and c-9, t-11 CLA isomers in CLA-50 and CLA-100, respectively). In the CLA-PM-105 DIM experiment, the aim was to achieve an individual dietary intake of 10 g/d of t-10, c-12 and c-9, t-11 CLA isomers in CLA-100, but von Soosten et al. (2011) reported that the animals of the CLA group consumed only 6 g/d of the t-10, c-12 and c-9, t-11 CLA isomers (40% lower than planned).

The unsaturated FA, which are preferentially saturated by ruminal microorganisms, must either be protected from ruminal biohydrogenation or be presented in amounts high enough to result in sufficient escape to the intestinal tract for absorption (Gillis et al., 2004a). In ruminant animals, FA composition of AT depots is dependent on: 1) supply of dietary FA to depots as influenced by the extent of ruminal biohydrogenation, as well as intestinal

absorption rates; 2) de novo synthesis of FA from precursors supplied to AT; and 3) rate of desaturation by the AT enzyme, Δ^9-desaturase (Enser et al., 1999). However, in the current study, another reason for the lack of a CLA effect could be that the uptake of the c-9, t-11 and t-10, c-12 CLA isomers by different tissues was insufficient to elicit metabolic effects. The effect of CLA supplementation on the FA distribution in different tissues has been reported in both experiments using the same animals as in the present study (Kramer et al., 2010, 2011). In the CLA-252 DIM experiment, Kramer et al. (2010) evaluated the effect of CLA supplementation on FA distribution of liver and SC AT (biopsy samples collected on d 21 AP, 1, 21, 70 and 105 PP) and reported that the FA distribution and FA ratios showed no significant differences between the CTR and the supplemented groups (CLA-50 and CLA-100). Thus, an effect of the CLA supplementation on the FA synthesis and Δ^9 desaturation in liver and AT could not be observed (Kramer et al., 2010). In the CLA-PM-105 DIM experiment, the FA distribution of liver tissue showed no significant changes between CTR and CLA supplement on d 105 PP. In contrast, retroperitoneal fat showed significant changes of c-9, t-11 (0.18% (CTR) to 0.24% (CLA) of FA methyl esters), and t-10, c-12 (0.00% (CTR) to 0.01% (CLA) of FA methyl esters) CLA isomers on d 105 PP (Kramer et al., 2011). Data on FA distribution and FA ratios for other fat tissues are not available yet.

4.3. Presence of haptoglobin in adipose tissue

The IHC method used in this study confirmed that Hp is present in all tested bovine AT, which is consistent with results obtained in mice (Friedrichs et al., 1995; Chiellini et al., 2002; do Nascimento et al., 2004), and in humans (Fain et al., 2004). Based on our results the presence of Hp is likely attributable to the adipocytes which is in agreement with Friedrichs et al. (1995), Chiellini et al. (2002), and do Nascimento et al. (2004). In contrast, Fain et al. (2004) reported that more Hp is made by the nonfat cells of human AT explants than is made by adipocytes. In the heifers investigated herein, the histological localization of Hp showed that VC fat had higher portions of Hp positive cells than SC fat. This finding is consistent with Fain et al. (2004) who reported that explants of human VC depots released more Hp than those of SC depots, and this might be due to the metabolic differences between the depots as mentioned previously in section 1.2.

The Western immunoblotting method further confirmed the presence of Hp in AT. This method demonstrated differences in the obtained molecular weights of Hp in SC tailhead fat

(26 and 55 kDa) as compared to the liver or serum (34, 26 and 20 kDa). This finding is in line with Dilda et al. (2011) who reported several isoforms of Hp in bovine gastric tissues (16 and 45 kDa in rumen and abomasum) that differed from those expressed in the liver (14 and 39 kDa). Furthermore, Cooray et al. (2007) mentioned that the bovine granulocyte Hp consists of α (20 kDa) and β (40 kDa) chains each containing four and five isoforms, respectively. The reason for these differences is not clear, but differences in the glycosylation patterns of this protein have the potential to change its molecular weight (Wilson et al., 2002). Furthermore, it is also possible that specific glycosylation patterns, for proteins in general, are expressed in a tissue-specific and developmentally regulated manner (Ervasti et al., 1997). However, further investigations are required to ensure the protein identity. Taken together, these findings (by IHC and Western immunoblotting methods) provide an indication that Hp could be classified as an adipokine in ruminants as reported in humans (Chiellini et al., 2004; Fain et al., 2004). Within AT, Hp can play a role as a monocyte chemoattractant factor (Maffei et al., 2009), and as an antioxidant or angiogenesis agent (do Nascimento et al., 2009). However, further studies are required to give insight on Hp functions in bovine AT.

It was suggested that Hp is also associated with obesity because its serum concentrations are increased in obese humans (Chiellini et al., 2004), and this is consistent with the concept that obesity is a state of chronic mild inflammation (do Nascimento et al., 2004). Unlike human studies, less is known about the relation between Hp and obesity in ruminants. In the current study, no relationship between serum Hp and each of BCS, BW and body fat content was established in both CLA experiments (not obese or over conditioned animals). In the over conditioned non-lactating heifers (fat heifers restriction study) there was a moderate negative correlation, which was completely surprising, between the concentrations of serum Hp and BW ($r = -0.51$, $P = 0.002$). Conversely, Chiellini et al. (2004) reported a strong positive relationship between circulating Hp and body fat in humans. The question as to whether the concentrations of Hp in serum might change in lactating cows if fat mass exceeds a certain level is unresolved yet. In addition, it is probable that Hp as an adiposity marker is irrelevant for ruminants.

4.4. Haptoglobin as an inflammation marker

Haptoglobin being one of the major APPs in cattle, is synthesized in response to infection and inflammation, with low constitutive serum concentrations under normal conditions ranging from 0.05 to 0.10 mg/mL (Svensson et al., 2007), exhibits a high relative increase up to 50 -

100-fold (Godson et al., 1996) and 1000-fold (Eckersall et al., 2006) in response to different inflammatory stimuli. Hirvonen et al. (1999) reported that the concentrations of Hp were high in cows with mastitis, metritis, and other inflammatory processes.

In the CLA studies, the clinical examination of the animals revealed disorders of their general health conditions such as mastitis, metritis and retained placenta. These diseases are considered as being of economic importance for dairy production (Kelton et al., 1998), and can dramatically affect the profitability of dairy herds (Kossaibati and Esslemont, 1997). Disease frequency is usually reported either as incidence (the occurrence rate of disease cases per unit of time) or prevalence (the proportion of animals that are diseased at any single point in time) (Kelton et al., 1998). For **mastitis,** the incidence rates in the CLA-252 DIM experiment were 0.24 and 0.15 cases/100 cow/d for cows and heifers, respectively, and the incidence rate in the CLA-PM-105 DIM experiment was 0.45 cases/100 cow/d. The incidence rate of heifers in the CLA-252 DIM experiment is consistent with that of Plym-Forshell et al. (1995) who reported 56 cases/100 cow/year (equivalent to 0.153 cases/100 cow/d) in Denmark, and Erskine et al. (1988) who reported an incidence rate of 4.23 cases/100 cow/month (equivalent to 0.141 cases/100 cow/d) in the USA. The incidence rates in our study in both experiments were higher than those reported by Gianneechini et al. (2002) (1.2 cases/100 cow/month) in Uruguay, and Plym-Forshell et al. (1995) (21 cases/100 cow/year) in Sweden, which as daily incidence can be estimated as 0.04 and 0.06 cases/100 cow/d, respectively. The incidence rates for **metritis** in the CLA-252 DIM experiment were 0.13 and 0.20 cases/100 cow/d for cows and heifers, respectively, whereas the incidence rate in the CLA-PM-105 DIM experiment was 0.45 cases/100 cow/d. These rates are considered low compared to Emanuelson et al. (1993) who reported 2.2 cases/100 cow/d in Sweden. For **retained placenta** in the CLA-252 DIM experiment, incidence rates of 0.04 and 0.03 cases/100 calvings for cows and heifers were recorded, respectively, while, in the CLA-PM-105 DIM experiment the incidence rate was 0.29 cases/100 calvings. These rates are considered low compared to Borsberry and Dobson (1989) who reported 1.3 cases/100 calvings in the UK.

The current results showed that the concentrations of Hp ranged from 0.01 to 12.4 mg/mL (n = 49, CLA-252 DIM experiment), 0.01 to 9.0 mg/mL (n = 25, CLA-PM-105 DIM experiment) and 0.02 to 3.5 mg/mL (n = 20, concentrate-roughage ratio study). The animals in the latter mentioned study were generally healthy, and they had significantly lower

concentrations of Hp compared to the animals in the CLA studies. This confirms that Hp is a useful indicator of general health status in animals. For the non-lactating heifers (n = 12, fat heifers restriction study), the concentrations of Hp ranged from 0.05 to 0.11 mg/mL.

Serum Hp concentrations were used to distinguish between healthy and diseased animals in the CLA-252 DIM experiment. A cut-off value of 0.16 mg/mL (59% sensitivity and 73% specificity) was obtained in the current study. Humblet et al. (2006) and Svensson et al. (2007) relate the poor sensitivity to the un-noticed subclinical health disturbances, which restricts the ability to identify animals with pathologic disorders. A cut-off value of 0.15 mg/mL (72% sensitivity and 59% specificity) was reported in dairy cattle suffering from respiratory-tract disease (Svensson et al., 2007), and a cut-off value of 0.19 mg/mL (98% sensitivity and 100% specificity) was established to differentiate between healthy cows and cows with different inflammatory diseases (Khoshvaghti et al., 2009). In the current CLA studies, 5% of the CLA-252 DIM samples (total 874), and 11% of the CLA-PM-105 DIM samples (total 209) had higher concentrations of Hp than 0.16 mg/mL without any clinical finding. It is to be mentioned that in the present study the time of diagnosis may not truly reflect the exact time of the disease onset. It should be considered that in the absence of disease, the animal health could be influenced by environmental factors, handling, and other types of stress (Petersen et al., 2004).

Chronic sub-clinical mastitis is a common problem in dairy herds with considerable economic losses, mainly due to reduced milk production and discarded milk. For successful mastitis control in a herd, rapid and accurate diagnosis of mastitic cows is crucial (Grönlund et al., 2005). Sub-clinical mastitis is mostly diagnosed by cow-side tests like the California Mastitis Test or by analyses of SCC using automatic cell counters. Acute phase proteins are sensitive and accurate parameters for mastitis diagnosis. In the CLA-PM-105 DIM experiment, from total milk samples (n = 96), 44% of the samples were below the detection limit of 0.07 µg/mL. The concentrations of Hp below the detection limit are considered as a good indicator of healthy udder quarters as reported by Grönlund et al. (2005). The concentrations of Hp in milk samples ranged from 0.07 to 0.74 µg/mL for healthy quarters and from 0.07 to 199 µg/mL for diseased quarters, which is consistent with Hiss et al. (2007), who reported that concentrations of Hp in milk ranged from 0.35 to 16 µg/mL in sterile samples and from 0.35 to 974 µg/mL in samples with pathogens. In addition, the SCC in milk is the most commonly used parameter in diagnosis of mastitis. Many studies reported the correlation between SCC

and APPs. In this study, a positive moderate correlation was found between the concentrations of milk Hp and SCC (r = 0.41, P = 0.001), while a very strong correlation (r = 0.80) was reported by Hiss et al. (2007). In the study of Hiss et al. (2007), to be able to include sufficient animals with subclinical mastitis, the cows were preselected on the basis of their milk SCC (i.e. half of the cows selected had elevated milk SCC for at least two months), which was not the case in the present study since the animals were not preselected depending on their SCC. This might be a proper reason that the correlation in our study is lower than in the study of Hiss et al. (2007).

4.5. Effect of fat mobilization on the concentrations of haptoglobin and leptin

The fat heifers restriction study aimed mainly at revealing if the change in EB induced any change in the concentrations of Hp and leptin in Simmental heifers fed grass silage or a hay/silage-straw mixture of reduced energy content. However, the current results indicated that neither Hp nor leptin concentrations were affected by the change in energy content of the ration fed. The results of serum Hp are in line with Berry et al. (2004) who demonstrated that two dietary energy levels (3.56 or 4.48 MJ NE for gain (NEg)/kg of feed on DM basis) and two dietary starch levels (34 or 48% of dietary ME from starch) fed to bull and steer calves for 42 days had no effect on the concentrations of serum Hp.

If leptin serves as an endocrine signal linking peripheral adipose stores to regulatory centers within the hypothalamus, changes in nutritional status should be accompanied by changes in the concentrations of serum leptin (Morrison et al., 2001). In sheep, restricted feeding (39% of the maintenance energy requirement (MER) for 3 days (Delavaud et al., 2000) and 38% of the MER for 14 weeks (Morrison et al., 2001) decreased the concentrations of plasma leptin. Also, in non-lactating, non pregnant Holstein cows initially fed at 130% of MER for 28 days, restricted to 21% of MER for 7 days, and re-fed to 237% of MER for 21 days, the concentrations of plasma leptin decreased by restricted feeding and increased after re-feeding (Delavaud et al., 2002). In the current study, the concentrations of leptin were not changed in animals fed hay/silage-straw mixture compared with animals fed grass silage. In addition, the re-feeding did not affect the concentrations of leptin in heifers fed hay/silage-straw mixture diet. Fasting has been reported to cause an inhibition of white AT leptin gene expression, which is tightly associated with decreasing leptin blood concentrations (Frühbeck, 2001). It is possible that the re-feeding causes an increase of leptin gene expression in white AT which is associated with an increase in the concentrations of serum leptin (Frühbeck, 2001). In view of

no effect of moderate feed restriction and re-feeding on the concentrations of Hp and leptin, it might be that the degree and the duration of feed restriction were not sufficient to induce any significant changes in the current study.

During fat mobilization, there is a concurrent loss of body condition and AT mass. The amount and rate of tissue mobilization probably depend on several factors such as the fatness of the cows, diet composition and extent of the energy deficit. High yielding cows have been selected and bred to produce more milk, mostly through their ability to mobilize fat and muscle to support milk production in early lactation (Knop and Cernescu, 2009). This results in a loss of body condition and is associated with alterations in blood metabolite and hormone profiles (Kadokawa and Martin, 2006). In this situation, the cows rely on the mobilization of adipose reserves and they often lose 60% or more of their body fat in the first weeks after parturition (Knop and Cernescu, 2009). As discussed before in section 4.1, in the Holstein cows the concentrations of Hp and leptin were increased and decreased around calving, respectively, and both were influenced by the onset of NEB and mobilization of AT. While, in the Simmental heifers the change in EB did not alter the concentrations of Hp and leptin. Also, using the same animals that were used in the current study, Germeroth et al. (2011) reported that Simmental heifers had larger adipocyte sizes in SC AT than Holstein heifers (CLA-PM-105 DIM experiment), indicating that the Simmental heifers tend to increase body reserves, whereas Holstein heifers spend extra energy preferentially for milk production. The concentrations of leptin in Simmental heifers used in this study were higher ($P < 0.001$) than that in Holstein heifers used in the CLA-PM-105 DIM experiment, probably due to the differing physiological state (lactating vs. non-lactating heifers), body fatness (BCS = 5 in Simmental heifers vs. 3 in Holstein heifers), and the nutritional conditions. Due to the higher concentrations of leptin and larger adipocyte sizes in the Simmental heifers compared with the Holstein heifers, it might be that the Simmental heifers display a lower attitude in mobilizing body reserve than the Holstein heifers.

5. Conclusions

The current study clearly demonstrated that the physiological changes in the concentrations of serum Hp were related to parity and parturition. In addition, heifers had higher concentrations of Hp in the first week PP than cows. This observation suggests a more intense physiological response to first calving than to later ones. Moreover, this study explored that the increase in the concentrations of Hp in the first week PP could be partly related to the metabolic stress, as indicated by the relation between the concentrations of Hp and each of NEFA and BHB. Furthermore, the concentrations of Hp showed a stronger positive relation with NEFA in heifers than in cows; therefore it might be that heifers are more stressed by lipolysis than cows.

Long-term supplementation with CLA (equal proportions of each c-9, t-11 and t-10, c-12 isomers) after calving in cows and heifers left the concentrations of serum Hp and leptin unaffected. Taken together, in this study it is difficult to make any definitive conclusions on the effect of long-term CLA supplementation on Hp and leptin. It is likely that dose and isomeric composition of CLA separately affect the ability of CLA to influence the concentrations of Hp and leptin in dairy cows. However, the results of this study might open new aspects for further studies to focus on the dose and single action of the c-9, t-10 and the t-10, c-12 CLA isomers on Hp and leptin.

The IHC and Western immunoblotting confirmed the presence of Hp in AT. This result gives an indication that Hp could be classified as an adipokine in cattle. Therefore, more attention should be paid to investigate the function of this protein to provide information as to why Hp is present in bovine AT. In addition, the lack of an evident relation between the concentrations of serum Hp and body fatness in the present study makes Hp irrelevant as an adiposity marker for ruminants.

Finally, it can be concluded that in response to NEB, Holstein cows mobilize stored triglycerides in the AT in an attempt to meet energy demands for maintenance and milk production. While, the Simmental heifers tend to increase body reserves and display a lower attitude in mobilizing body reserve than the Holstein cows.

6. Summary

Timely changes of the circulating concentrations of haptoglobin and leptin in female cattle undergoing fat mobilization: assessment of the relevance of haptoglobin as an adipokine

Dairy cattle production exposes animals to a variety of stressors which can reduce feed intake and disease resistance. During the transition period (3 weeks AP to 3 weeks PP), the energy requirements can not entirely be met through dietary intake resulting in a condition of NEB, and cows may experience adverse health events. Extensive metabolic and endocrine changes occur in dairy cows to accommodate parturition and lactogenesis. Dairy cows during this period are susceptible to various metabolic diseases, impaired immune function and fertility. Dairy cattle production has been looking for more efficient and effective ways to minimize these health problems for a long time. In the last decade, there was sharply increasing interest in CLA due to its potentially beneficial effects in terms of animal welfare, nutritional status and diseases. Haptoglobin, one of the major APPs in cattle, is synthesized in response to infection and inflammation. In healthy cattle, Hp blood concentrations are low or even undetectable, but increase up to 1000-fold in response to different inflammatory stimuli. Leptin is a protein secreted from AT and has been implicated in regulation of food intake, energy expenditure and whole-body EB. However, circulating concentrations of Hp and leptin under long-term CLA supplementation have not yet been investigated. In addition, less is known about other sites of Hp production in ruminants such as AT. Moreover, as early lactating cows favor mobilization of AT under conditions of NEB, the initial step in non-lactating cows to overcome the energy deficit is the mobilization of an excessive quantity of AT. Therefore, this study was undertaken to characterize the concentrations of Hp and leptin throughout the entire lactation period or during early lactation, to investigate the potential effects of CLA supplementation on the concentrations of Hp and leptin in dairy cows, to examine the presence of Hp in bovine AT, and to examine the effect of moderate feed restriction and re-feeding on the concentrations of Hp and leptin in non-lactating, non-pregnant heifers.

Samples from four different studies were used in this work. The first experiment **"CLA-252 DIM"** was conducted to characterize the concentrations of Hp and leptin throughout lactation and to evaluate the effects of two different doses of rumen-protected CLA (50 and 100 g/d) supplemented from d 1 to 182 PP. This experiment comprised heifers (n = 16) and cows (n =

33) observed from d 21 AP until d 252 PP. In addition, a further experiment **"CLA-PM-105 DIM"** was done in heifers (n = 25) studied from d 21 AP until d 105 PP and received 100 g/d CLA supplementation. The animals were sequentially slaughtered (d 1, 42 or 105 PP) for collection of PM tissue samples (3 VC and 3 SC depots). The CLA supplementation started on d 1 PP and continued until slaughter. In both experiments, the CLA supplementation included 12% (of total FA) of the t-10, c-12 and c-9, t-11 CLA isomers in equal parts. The third experiment **"Concentrate-roughage ratio"** was conducted using cows (n = 20) investigated from d 21 AP until d 21 PP, and received diets after calving with either a concentrate-to-roughage ratio on a dry matter basis of 30:70% (low-concentrate group) or 60:40% (high-concentrate group). To examine the effect of moderate feed restriction and re-feeding, a fourth experiment **"Fat heifers restriction"** with 12 non-lactating, non-pregnant heifers was performed. The heifers were fed either grass silage (100%) or grass silage diluted with straw (37:63 on DM basis). Serum and milk Hp, and serum leptin concentrations in the collected samples were detected using a competitive ELISA. Haptoglobin in AT was detected using IHC and Western immunoblotting.

The physiological changes of Hp observed during the CLA-252 DIM experiment were related to parity (heifers vs. cows; $P < 0.05$) and parturition (before vs. after parturition; $P < 0.001$). The concentrations of Hp were 1.7-fold higher in heifers than in cows during the first week PP. The concentrations of Hp peaked around calving and decreased afterwards for both parity groups. In the CLA-PM-105 DIM experiment, the concentrations of Hp in heifers were affected by parturition ($P < 0.001$) with elevated concentrations during the first week PP and decreased afterwards. In both experiments, the time course of the concentrations of Hp after the first week PP did not show marked changes. Long-term feeding of dairy cows with CLA had no significant effect on the concentrations of serum Hp. No relationship was established between serum Hp and each of BCS, BW and body fat content in both CLA experiments. A positive relation was observed between the concentrations of Hp and NEFA in both CLA experiments, and this relation was stronger in heifers than in cows. In both CLA studies, the clinical examination of the animals revealed disorders of their general health conditions such as mastitis, metritis and retained placenta. The incidence rates of these diseases were determined. A cut-off point of 0.16 mg/mL with 59% sensitivity and 73% specificity was determined for the concentrations of serum Hp in the CLA-252 DIM experiment to discriminate between healthy and diseased animals. The IHC confirmed that Hp is present in all tested bovine AT. The VC fat had higher ($P < 0.05$) portions of Hp positive cells than SC fat. The Western immunoblotting indicated the presence of Hp in AT, and demonstrated

differences in the obtained molecular weights of SC tailhead fat (26 and 55 kDa) compared to liver or serum (34, 26 and 20 kDa).

The physiological alterations in the concentrations of serum leptin in the CLA-252 DIM experiment were limited to parturition (before vs. after parturition; $P < 0.001$), whereas no parity (heifers vs. cows) differences could be confirmed. In both CLA studies, the concentrations of leptin were high in the AP period, and then decreased in the PP period below the pregnancy concentrations. Long-term feeding of dairy cows with CLA left the concentrations of serum leptin unaffected. The concentrations of leptin had a stronger relation with fat mass, body fat content, and adipocyte size than BCS.

The fat heifers restriction study indicated that neither Hp nor leptin concentrations were affected by the change in energy content of the ration fed. The re-feeding (hay/silage-straw mixture to grass silage) had no significant effect on the concentrations of leptin.

In conclusion, the physiological changes observed in the CLA-252 DIM experiment in the concentrations of serum Hp were related to parity and parturition. During the first week PP, the concentrations of Hp were higher in heifers than in cows, suggesting a more intense physiological response to first calving than to later ones. This study explored that the metabolic stress might modulate part of the increase in the concentrations of Hp in the first week PP as indicated by the relation between the concentrations of Hp and NEFA. Long-term feeding of dairy cows with CLA (equal proportions of each the c-9, t-11 and the t-10, c-12 isomers) had no significant effect on the concentrations of serum Hp and leptin. However, the c-9, t-10 and the t-10, c-12 CLA isomer might divergently affect Hp and leptin. The presence of Hp in bovine AT indicates that Hp can be classified as an adipokine in cattle. The lack of an evident relation between the concentrations of Hp and body fatness in this study makes Hp irrelevant as an adiposity marker for ruminants.

7. Zusammenfassung

Zeitlicher Verlauf der zirkulierenden Konzentrationen von Haptoglobin und Leptin in weiblichen Rindern, die eine Fettmobilisierung durchleben: Einschätzung der Relevanz von Haptoglobin als Adipokin

Milchproduktion setzt die Tiere einer Vielzahl von Stressoren aus, die zu reduzierter Futteraufnahme und verminderter Krankheitsresistenz führen können. In der Transitionsperiode (3 Wochen vor der Geburt bis 3 Wochen nach der Geburt) können die Energieanforderungen durch die Futteraufnahme nicht mehr gedeckt werden, was zu einer NEB führt; dadurch sind Kühe nachteiligen Gesundheitsveränderungen ausgesetzt. Folglich resultieren daraus metabolische und endokrine Veränderungen um die Geburt und Milchproduktion zu gewährleisten. Milchkühe in diesem Zeitraum sind anfällig für eine Reihe metabolischer Erkrankungen, verminderter Immunfunktion und reduzierte Fruchtbarkeit. In der Produktion im Milchviehsektor wird schon seit langem nach effektiven Wegen gesucht, um die Gesundheitsprobleme der Kühe in dieser Zeit zu minimieren. Im letzten Jahrzehnt hat das Interesse an CLA zugenommen auf Grund deren potentiell positiver Wirkung auf das Wohlbefinden des Tieres, den Fütterungszustand und in Bezug auf Krankheiten. Haptoglobin ist eines der wichtigsten Akute Phase Proteine bei Rindern das als Antwort auf Infektion und Entzündung produziert wird. Bei gesunden Rindern sind die Blut Hp-Konzentrationen sehr niedrig bis nicht detektierbar, aber auf inflammatorische Stimulation können sie um das 1000-fache ansteigen. Leptin ist ein Protein, das vom Fettgewebe produziert wird, es ist in die Regulation der Futteraufnahme involviert und gibt Auskunft über den Energiezustand und die Energiebilanz des Körpers. Zirkulierende Konzentrationen von Hp und Leptin unter längerer CLA-Supplementierung wurden noch nicht untersucht. Außerdem ist wenig bekannt über andere Gewebe die Hp in Wiederkäuern synthetisieren, wie das Fettgewebe. Außerdem begünstigen die ersten Tage der Laktation einer Kuh, die Fettmobilisierung aus dem Fettgewebe unter NEB, der erste Schritt nicht laktierender Kühe das Energiedefizit zu überwinden ist die exzessive Mobilisierung von Fett.

Diese Studie wurde durchgeführt, um die Konzentrationen von Hp und Leptin über die komplette Laktation und über die ersten Tage der Laktation zu charakterisieren, den potentiellen Effekt der CLA-Supplementation auf Hp- und Leptinkonzentrationen in Milchkühen zu erforschen, das Vorkommen von Hp in bovinem Fettgewebe und den Effekt

von moderater Futterrestriktion und Wiederaufnahme der Fütterung in nicht laktierenden, nicht tragenden Färsen zu zeigen.

Proben aus vier verschiedenen Versuchen wurde für diese Studie verwendet. Der erste Versuch **"CLA-252 DIM"** wurde durchgeführt, um die Konzentrationen von Hp und Leptin über eine Laktation zu charakterisieren und um die Effekte der Fütterung von zwei verschiedenen Dosen pansengeschützer CLA (50 und 100 g/d) zu evaluieren, die von d 1 bis d 182 nach der Geburt gefüttert wurden. Dieser Versuch umfasste Färsen (n = 16) und Kühe (n = 33), die von d 21 bis d 252 nach der Geburt untersucht wurden. Zusätzlich wurde ein weiterer Versuch **"CLA-PM-105 DIM"** mit Färsen (n = 25) durchgeführt, die 100 g CLA/d gefüttert bekamen. Die Tiere wurden nacheinander für die Gewinnung von post mortalen Gewebeproben (3 viszerale und 3 subkutane Fett-depots) geschlachtet (d 1, 42 oder 105 PP). Die CLA Supplementierung begann an d 1 nach der Geburt bis hin zur Schlachtung. In beiden Versuchen enthielten die zugefütterten CLA zu je 12% (der Gesamtfettsäuren) die t-10, c-12 und die c-9, t-11 Isomere. Der dritte Versuch **"Kraftfutter Rauhfutter Verhältnis"** (KF:R) (d 21 AP - d 21 PP) wurde mit Kühen durchgeführt, deren Rationen bezogen auf Trockenmasse geringe (KF:R = 30:70%) oder hohe (KF:R = 60:40%) KF-Anteile nach der Kalbung enthielten. Um den Effekt von moderater Futterrestriktion und Wiederfütterung zu untersuchen, wurde ein vierter Versuch **"Fette Färsen"** mit 12 nicht laktierenden, nicht tragenden Färsen durchgeführt. Die Färsen bekamen entweder Grassilage (100%) oder Grassilage verdünnt mit Stroh (37:63 bezogen auf Trockenmasse). Serum und Milch Hp- und Serum-leptinkonzentrationen wurden mittels kompetitivem ELISA bestimmt. Haptoglobin in Fettgewebe wurde mit Immunhistochemie und Western Blot detektiert.

Die physiologischen Veränderungen von Hp im CLA-252 DIM Versuch standen im Zusammenhang zu Parität (Färsen vs. Kühe; $P < 0,05$) und Geburt (vor vs. nach der Geburt; $P < 0,001$). Die Konzentration von Hp bei Färsen war 1,7-fach höher als bei Kühen in der ersten Woche nach der Geburt. In beiden Paritätsgruppen und CLA Versuchen konnte gezeigt werden, dass Hp eine maximale Konzentration zum Zeitpunkt der Geburt hatte und anschließend wieder absank. Im CLA-PM-105 DIM Versuch war die Hp-Konzentration in Färsen durch die Geburt beeinflusst ($P < 0,001$), erhöhte Konzentrationen wurden in der ersten Woche nach der Geburt gemessen, die danach wieder absanken. In beiden Versuchen zeigte der zeitliche Verlauf der Hp-Konzentration nach der ersten Woche PP keine auffälligen Veränderungen. Die CLA-Fütterung von Milchkühen über einen längeren Zeitraum zeigte keine signifikanten Effekte auf die Hp-Konzentration. In beiden CLA-Gruppen konnte kein

Zusammenhang zwischen Serum-Hp und BCS, Körpergewicht und Körperfettanteil ermittelt werden. Ein positiver Zusammenhang konnte zwischen der Hp- und NEFA-Konzentration in beiden CLA-Versuchen ermittelt werden, die Beziehung bei den Färsen war stärker als die bei den Kühen. In beiden CLA-Studien wurden durch klinische Untersuchungen verschiedener Erkrankungen, wie Mastitis, Metritis und retinierter Plancenta, festgestellt. Die Häufigkeitsraten dieser Erkrankungen wurden notiert. Ein Cut-off-Wert von 0,16 mg Hp/mL mit 59% Sensitivität und 73% Spezifität im CLA-252 DIM Versuch wurde ermittelt, um zwischen gesunden und kranken Tieren zu differenzieren.

Die IHC bestätigte, dass Hp in allen getesteten Fettgeweben der Kuh vorkommt. Im viszeralen Fettgewebe wurden mehr Hp-postitive Zellen ($P < 0,05$) gefunden als im subkutanen. Durch Western Blot konnte das Vorkommen von Hp im Fettgewebe und unterschiedliche Molekulargewichte des Haptoglobins im subkutanen Schwanzfett (26 und 55 kDa) verglichen mit Leber und Serum (34, 26 und 20 kDa) gezeigt werden. Die physiologischen Veränderungen in der Serum-Leptinkonzentration im CLA-252 DIM Versuch waren beschränkt auf die Geburt (vor vs. nach der Geburt; $P < 0,001$) wohingegen keine Paritätsunterschiede (Färsen vs. Kühe) festgestellt werden konnten. In beiden CLA-Versuchen war die Leptinkonzentration in der Zeit AP hoch und sanken in der Zeit PP bis unter die Konzentration während der Trächtigkeit ab. Langzeitsupplementation von Kühen mit CLA hatte keinen Einfluss auf die Serum-Leptinkonzentration. Die Leptinkonzentration stand in stärkerer Beziehung zur Köperfettmasse, Köperfettanteil und der Adipozytengröße als zum BCS. Die “Fette Färsen” Studie zeigte, dass weder Hp- noch Leptinkonzentrationen durch Änderungen im Energiegehalt der Ration beeinflusst wurden. Wiederaufnahme der Fütterung (Grassilage vs. Heu/Grassilage-Stroh-Mischung) hatte keinen Effekt auf die Leptinkonzentration.

Folglich waren die physiologischen Veränderungen in der Serum-Hp-Konzentration, die im CLA-252 DIM Versuch beobachtet wurden, auf die Parität und Geburt zurückzuführen. Während der ersten Woche PP waren die Hp-Konzentrationen in Färsen höher als in Kühen, was auf eine intensivere physiologische Antwort auf die erste Geburt als auf die Späteren hinweist. In dieser Studie wurde gezeigt, dass metabolischer Stress zumindest teilweise für einen Anstieg der Hp-Konzentrationen in der ersten Woche nach der Geburt verantwortlich ist, was über eine Beziehung zwischen Hp- und NEFA-Konzentration erklärt werden kann. Langzeitfütterung von Kühen mit einer CLA-Mischung, die gleiche Teile der c-9, t-11 und t-10, c-12 Isomere enthält, hatte keinen signifikanten Einfluss auf die Serum-Konzentrationen

von Hp und Leptin. Dennoch könnten die c-9, t-11 und t-10, c-12 Isomere Hp und Leptin unterschiedlich beeinflussen. Das Vorkommen von Hp in bovinem Fettgewebe indiziert, dass Hp bei Rindern als Adipokin klassifiziert werden kann. Der fehlende Zusammenhang zwischen Hp und Körperfettgehalt macht Hp irrelevant als Marker für Fettleibigkeit in Wiederkäuern.

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9. Appendixes

Appendix A - Animal diets

Tab. A1. Ingredients and chemical composition of the ante partum diet in the CLA-252 DIM experiment.

	PMR[1]		Concentrate
	Corn silage	Grass silage	
Components (%)			
Wheat grain			25
Barley grain			25
Soybean meal			20
Sugar beet pulp (dried)			11
Zeolithe[2]			12.5
Vitamin/mineral premix[3]			6.5
Chemical composition			
DM (g/kg)	295	457	877
Nutrients (g/kg of DM)			
Crude ash	40	106	83
Crude protein	66	113	179
Ether extract	31	32	31
ADF	238	332	66
NDF	445	555	189
Energy[4] (MJ/Kg DM)			
ME	10.6	9.2	10.6
NE_L	6.4	5.4	6.7

[1]Partial mixed ration on DM basis (60% corn silage, 40% grass silage).
[2]According to Grabherr et al. (2009) cows received 250 g Zeolithe/d for prevention of hypocalcaemia.
[3]Per kg mineral feed: 60 g Ca, 105 g Na, 80 g P, 50 g Mg, 7 g Zn, 5.4 g Mn, 1.25 g Cu, 100 mg I, 40 mg Se, 30 mg Co, 800 000 IU vitamin A, 100 000 IU vitamin D_3, 1500 mg vitamin E.
[4]Calculation based on nutrient digestibilities measured with wethers (GfE, 1991) and tabulated values (Universität Hohenheim-Dokumentationsstelle, 1997).
Abbreviations: ADF = Acid detergent fiber, DM = Dry matter, ME = Metabolizable energy, NDF = Neutral detergent fiber, NE_L = Net energy lactation.

Tab. A2. Ingredients and chemical composition of post partum fed concentrate and partial mixed ration in the CLA-252 DIM experiment (modified after Pappritz et al., 2011).

	Concentrate		PMR[1]
	CTR	CLA	
Ingredients (%)			
Wheat grain	38.5	38.5	
Sugar beet pulp (dried)	29	29	
Rapeseed meal	20	20	
Soybean meal	6.5	6.5	
Soybean oil	1	1	
Calcium carbonate	0.50	0.50	
Vitamin/mineral premix[2]	2	2	
CLA supplement		2.5	
Control fatty acid	2.5		
Analyzed chemical profile			
DM (g/kg)	889	887	426
Nutrients (g/kg DM)			
Crude ash	71	74	69
Crude protein	187	187	118
Ether extract	59	53	32
Crude fiber	88	89	193
ADF	123	124	225
NDF	258	256	425
Energy[3] (MJ/Kg DM)			
ME	13.9	13.8	11.1
NE_L	8.8	8.8	6.8
CLA (g/kg of DM)			
t-10, c-12 CLA	0.02	2.25	0.01

[1]Partial mixed ration (63% silage, 37% PMR-concentrate on DM basis).

[2]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 D_3, 1500 mg Vitamin E.

[3]Calculation based on nutrient digestibilities measured with wethers (GfE, 1991).

Abbreviations: ADF = Acid detergent fiber, c = cis, CLA = Conjugated linoleic acids, DM = Dry matter, ME = Metabolizable energy, NDF = Neutral detergent fiber, NE_L = Net energy lactation, t = trans.

Tab. A3. Fatty acids profile of fat supplements[1] in the CLA-252 DIM and the CLA-PM-105 DIM experiments (modified after Pappritz et al., 2011; von Soosten et al., 2011).

Fatty acids (% of total fatty acids)	CTR	CLA
Palmitic acid (C16:0)	10.89	10.89
Stearic acid (C18:0)	87.30	50.31
Oleic acid (C18:1 c-9)	<0.01	10.66
Conjugated linoleic acids (CLA)		
C18:2 c-9, t-11	0.06	11.99
C18:2 t-10, c-12	0.02	11.88
Other CLA	0.15	0.95
Other fatty acids	1.58	3.32

[1]Supplementation CLA was included in the concentrate portion (fed by the computerized concentrate feeding station) as a rumen-protected CLA preparation. For the control group, CLA was distributed by stearic acid (C18:0).

Tab. A4. Ingredients and chemical composition of the ante partum diet in the CLA-PM-105 DIM experiment (von Soosten et al., 2011).

	Concentrate	PMR[1]
Ingredients (%)		
Wheat grain	25	
Barley grain	25	
Soybean meal	20	
Sugar beet pulp (dried)	11	
Zeolithe[2]	12.5	
Vitamin/mineral premix[3]	6.5	
Chemical composition		
DM (g/kg)	894	375
Nutrients (g/kg DM)		
Crude ash	155	56
Crude protein	173	89
Ether extract	22	29
Crude fiber	51	232
ADF	68	256
NDF	157	469
Energy[4] (MJ/kg DM)		
ME	10.6	11.0
NE_L	6.7	6.7

[1]Partial mixed ration on DM basis (60% corn silage, 40% grass silage).

[2]According to Grabherr et al. (2009) cows received 250 g Zeolithe/d for prevention of hypocalcaemia.

[3]Per kg mineral feed: 60 g Ca, 105 g Na, 80 g P, 50 g Mg, 7 g Zn, 5.4 g Mn, 1.25 g Cu, 100 mg I, 40 mg Se, 30 mg Co, 800 000 IU vitamin A, 100 000 IU vitamin D_3, 1500 mg vitamin E.

[4]Calculation based on nutrient digestibilities measured with wethers (GfE, 1991) and tabulated values (Universität Hohenheim-Dokumentationsstelle, 1997).

Abbreviations: ADF = Acid detergent fiber, DM = Dry matter, ME = Metabolizable energy, NDF = Neutral detergent fiber, NE_L = Net energy lactation.

Tab. A5. Ingredients and chemical composition of post partum concentrate and partial mixed ration in the CLA-PM-105 DIM experiment (von Soosten et al., 2011).

	Concentrate			PMR[1]
	CTR	CLA	PMR[1]	
Ingredients (%)				
Wheat grain	39.5	39.5	41	
Sugar beet pulp (dried)	29	29	30	
Rapeseed meal	20	20	20	
Soybean meal	6.5	6.5	6.5	
Vitamin/mineral premix[2]	2	2	2	
Control fat supplement	2.5	-	-	
CLA supplement	-	2.5	-	
Calcium carbonate	0.5	0.5	0.5	
Analyzed chemical profile				
DM (g/kg)	873	871	870	445
Nutrients (g/kg DM)				
Crude ash	65.2	69	64	62
Crude protein	182	180	182	124
Ether extract	50	44	20	28
ADF	134	133	134	208
NDF	259	260	265	405
Energy[3] (MJ/kg DM)				
ME	13.9	13.7	13.5	11.9
NE_L	8.9	8.7	8.7	7.5
CLA (g/kg of DM)				
C18:2 t-10, c-12	0.0	1.7	0.0	0.0
C18:2 c-9, t-11	0.0	1.6	0.0	0.0

[1]Partial mixed ration (25% grass silage, 38% corn silage, 37% PMR-concentrate).
[2]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 D_3, 1500 mg vitamin E.
[3]Calculation based on nutrient digestibilites measured with wethers (GfE, 1991).
Abbreviations: ADF = Acid detergent fiber, c = cis, CLA = Conjugated linoleic acids, DM = Dry matter, ME = Metabolizable energy, NE_L = Net energy lactation, NDF = Neutral detergent fiber, t = trans.

Tab. A6. Nutrient, fibre and energy contents of the feed ingredients as well as intended percentages of silage and concentrate in the diets fed to cows post partum in the concentrate-roughage ratio study (Locher et al., 2011).

	Feeding component			
Item	Corn silage	Grass silage	LC	HC
DM (g/kg)	368	289	885	883
Ash (g/kg DM)	37	138	53	66
CP (g/kg DM)	80	159	218	210
EE (g/kg DM)	32	40	29	27
ADF (g/kg DM)	223	311	52	53
NDF (g/kg DM)	424	514	146	163
ME (MJ/kg DM)	10.7[1]	10.8[1]	13.1[2]	12.9[2]
NE_L (MJ/kg DM)	6.4[1]	6.5[1]	8.3[2]	8.2[2]
% in LC diet	42	28	30	-
% in HC diet	24	16	-	60

[1]Calculation based on nutrient digestibilities measured with wethers (GfE, 1991).

[2]Calculation based on analyzed nutrient contents and tabulated values of apparent digestibilities (Universität Hohenheim-Dokumentationsstelle, 1997).

Abbreviations: ADF = Acid detergent fiber, CP = Crude protein, DM = Dry matter, EE = Ether extract, HC = High concentrate, LC = Low concentrate, ME = Metabolizable energy, NDF = Neutral detergent fiber, NE_L = Net energy lactation.

Tab. A7. Chemical composition and energy contents of grass silage and hay/silage-straw mixture in the fat heifers feed restriction study (unpublished data).

	Feeding component	
Item	Grass silage	Hay/silage-straw mixture
DM (%)	37	63
CP (%)	10.6	8.5
ADF (%)	34.6	37
NDF (%)	54.8	63.2
ME (MJ/kg DM)	9.5	8.1

Abbreviations: ADF = Acid detergent fiber, CP = Crude protein, DM = Dry matter, ME Metabolizable energy, NDF = Neutral detergent fiber.

Appendix B - Correlations

Tab. B1. Coefficients of correlation between the concentrations of serum haptoglobin and body condition score, body weight, concentrations of nonesterified fatty acids and β-hydroxybutyrate during different periods in the conjugated linoleic acid studies.

		Cows				Heifers			
Experiment	Period	BCS	BW	NEFA	BHB	BCS	BW	NEFA	BHB
CLA-252 DIM	21 AP - 252 PP	-	-	0.14***	0.12***	-	-	0.38***	-
	21 AP - 7 AP	-	-	-	-	-	-	-	-
	1 PP - 252 PP	-	-	0.10*	0.11*	-	-	0.43***	-
	1 PP - 182 PP	-	-	0.12*	0.17**	-	-	0.56***	-
	189 PP - 252 PP	-	-	0.26***	-	-	-	-	-
CLA-PM-105 DIM	21AP - 105 PP					-	-	0.54***	0.20**
	21 AP - 7 AP					-	-	0.46***	-
	1 PP - 105 PP					-	-	0.49***	0.23**

* P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.
Abbreviations: AP = Ante partum, BCS = Body condition score, BHB = β-hydroxybutyrate, BW = Body weight, NEFA = Nonesterified fatty acids, PP = Post partum.

Tab. B2. Coefficients of correlation between the concentrations of serum leptin and body condition score, body weight, concentrations of nonesterified fatty acids and β-hydroxybutyrate during different periods in the CLA-252 DIM and CLA-PM-105 DIM experiments.

		All animals				Heifers			
Experiment	Period	BCS	BW	NEFA	BHB	BCS	BW	NEFA	BHB
CLA-252 DIM	21 AP - 252 PP	0.19***	-	-0.15***	-				
	21 AP - 7 AP	-	-	-	-				
	1 PP - 252 PP	-	-	-0.11**	-				
	1 PP - 182 PP	-	-	-0.10*	-				
	189 PP - 252 PP	0.18**	-	-	-				
CLA-PM-105 DIM	21AP - 105 PP	-	-	-	-	-	-	-	-
	21 AP - 7 AP	-	-	-	-	0.27*	-	-	-
	1 PP - 105 PP	-	-	-	-	0.34***	0.46***	-	0.34***

* P ≤ 0.05, ** P ≤ 0.01, *** P ≤ 0.001.
Abbreviations: AP = Ante partum, BCS = Body condition score, BHB = β-hydroxybutyrate, BW = Body weight, NEFA = Nonesterified fatty acids, PP = Post partum.

Tab. B3. Coefficients of correlation between the concentrations of serum leptin and fat mass, adipocyte size and body fat content of different fat depots for the heifers in the CLA-PM-105 DIM experiment.

	Fat mass (kg)			Adipocyte size (μm^2)			Body fat content (%)		
	1 PP	42 PP	105 PP	1 PP	42 PP	105 PP	1 PP	42 PP	105 PP
SC depots									
Tailhead	-	-	-	-	-	-	-	-	-
Withers	-	-	-	-	-	0.78**	-	-	-
Sternum	-	-	-	-	-	0.71*	-	-	-
Mean SC	-	0.83**	-	-	-	-	-	0.80**	-
VC depots									
Omental	-	0.90***	-	-	-	-	-	0.86***	-
Mesenterial	-	0.71*	-	0.98**	-	-	-	0.72*	-
Retroperitoneal	-	0.81**	-	-	-	0.75*	-	0.83**	-

* $P \leq 0.05$, ** $P \leq 0.01$, *** $P \leq 0.001$.
Abbreviations: PP = Post partum, SC = Subcutaneous, VC = Visceral

Appendix C - Chemicals, buffers and solutions

Enzyme-linked immunosorbent assay (ELISA)

Protease inhibitor Complete™	(Roche)
1 tablet in 2 mL H_2O	
Coating buffer	
0.05 M $NaHCO_3$	(Roth)
200 µL/L Protease inhibitor	
20 mL/L Proclin 150®	(Sigma)
pH 9.6	
Casein solution	
0.05 M NaOH	(Applichem)
1.5 mM EDTA (Disodium salt dihydrate)	(Roth)
2.5% Casein	(Sigma)
200 µL/L Protease inhibitor	
20 mL/L Proclin 150®	
pH 7.2	
Test buffer	
0.12 M NaCl	(Roth)
0.02 M Na_2HPO_4	(AppliChem)
0.01 M EDTA	(Roth)
0.005% Chlorhexidine	(Sigma)
0.1% Gelatine (Gelatine Hydrolysate)	(Sigma)
0.05% Tween®20	(AppliChem)
0.002% Phenol red	(Sigma)
200 µL/L Protease inhibitor	
20 mL/L Proclin 150®	
pH 7.3	
Substrate buffer	
0.05 M Citric acid	(AppliChem)
0.055 M Na_2HPO_4	
0.05% Urea peroxide	(Sigma)
20 mL/L Proclin 150®	
pH 4.05	
TMB-solution	
12.5 mg TMB (3, 3, 5, 5-Tetramethylbenzidine)	(AppliChem)
1 mL DMSO (Dimethyl sulfoxide)	(AppliChem)
Substrate solution	
18 mL Substrate buffer	
360 µL TMB-solution	

Stop solution
1 M Oxalic acid (Roth)

Washing buffer (10-fold)
1.36 M NaCl
81 mM Na_2HPO_4
27 mM KCl (Roth)
15 mM KH_2PO_4 (Roth)
20 mL/L Proclin 150®
55 g/L Tween®20
pH 7.3
To prepare a ready-made solution: 10 mL of washing buffer/1 L

Immunohistochemistry (IHC)

PBS (Phosphate buffered saline)
0.136 M NaCl
8.1 mM Na_2HPO_4
2.7 mM KCl
1.5 mM KH_2PO_4
pH 7.2

Washing buffer
0.136 M NaCl
8.1 mM Na_2HPO_4
2.7 mM KCl
1.5 mM KH_2PO_4
0.5 ml (0.05%) Tween®20
pH 7.2
Filled up with H_2O (1 L)

Citrate buffer
0.01 mM Citric acid, Anhydrous
pH 6
Filled up with H_2O (1 L)

Hydrogen peroxide (H_2O_2)
100 µL H_2O_2 (Roth)
1 mL PBS

Western immunoblotting

Resolving buffer
1.5 M Tris (hydroxymethyl aminomethane)/HCl (Roth)
0.4% SDS (Sodium dodecyl sulfate) (Sigma)
pH 8.8

Resolving gel (12%)
4 mL Acrylamide (30%) (Rotiphorese® Gel A) (Roth)
1.6 mL Bisacrylamide (2%) (Rotiphorese® Gel B) (Roth)

2.50 mL Resolving buffer
1.9 mL H_2O
The above mixture mixed with:
45 µL 10% Ammonium persulfate (APS) (Sigma)
5 µL Tetramethylenediamine (TEMED) (Roth)

Stacking buffer
0.5 M Tris/HCl
0.4% SDS
pH 6.8

Stacking gel (5.6%)
0.40 mL Acrylamide (30%)
0.265 mL Bisacrylamide (2%)
0.625 mL Stacking buffer
1.71 mL H_2O
The above mixture mixed with:
20 µL 10% APS
5 µL TEMED

Running buffer (10-fold)
25 mM Tris (Roth)
0.2 M Glycine
0.1% SDS

Bromphenol blue solution
0.2% Bromophenol blue (Sigma)
0.1 M Tris/HCl
pH 7.5

Sample buffer (2-fold)
3 mL Bromophenol blue solution
40 mL Glycerine (Merck)
4 mL 2-Mercaptoethanol (Sigma)
10 mL 20% SDS
4 mL 1M Tris/HCl
pH 6.8
Filled up with H_2O (1 L),
for 5-fold filled up with 40 mL H_2O

Tris/HCl
0.1 M Tris/HCl
pH 7.5

Blotting buffer I
0.3 M Tris/HCl
10% methanol (AppliChem)
pH 10.4

Blotting buffer II
25 mM Tris/HCl
10% methanol
pH 10.4

Blotting buffer III
25 mM Tris/HCl
10% methanol
60 mM 6-Aminohexanoic acid
pH 9.4

TBS buffer (10-fold)
0.05 M Tris/HCl
0.9% NaCl
pH 7.5

TBST (Tris-Buffered Saline-Tween)
0.5 mL (0.05%) Tween®20
1 L TBS
pH 7.3-7.4

TBST with Roti®block
9 mL TBST
1 mL Roti® block

Fat and liver samples homogenization

Homogenization buffer
50 mM Tris HCl
300 mM KCl
2.5 mM $MgCl_2$
pH 7

Protease inhibitor Complete™
1 tablet in 2 mL H_2O

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