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Characterization of insulin sensitivity and inflammation related factors in dairy cows receiving conjugated linoleic acids (CLA) or a control fat supplement during lactation

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Dedicated to my family, specially my wonderful wife
because of her endless love and encouragements and patiently tolerating all the extra hours I had always spent on my research works

English abstract

With the onset of lactation, dairy cows have to mobilize body reserves, mainly body fat, to cover the output of energy via milk. The homeorhetic metabolic adaptation to the needs of milk production is accomplished through the orchestrated action of hormones. In contrast to the "classical hormones" that knowingly control parturition, lactation and metabolism, the role and importance of messenger molecules originating from body fat (adipokines), of their receptors and also of nuclear receptors as key regulators of gene expression was only scarcely investigated in dairy cows. In particular, data on body fat were largely limited to subcutaneous (s.c.) fat from one location easily accessible via biopsy, whereas potentially heterogeneous reactions between different s.c. depots and also in different visceral (v.c.) fat were not yet comprehensively addressed. The aim of this dissertation was to characterize the mRNA expression of several adipokines and related factors that are involved in insulin sensitivity (IS) and in inflammation during the transition from pregnancy to lactation and during the subsequent lactation. In addition, dietary supplementation with either CLA vs. a control fat (supplementation period day 1 to day 105 or 182 of lactation) was tested for potential effects on the target mRNAs. The tissue in focus was adipose tissue (AT) with its different locations. Initially, suitable reference genes were identified as a methodological prerequisite for the studies. Using tissue samples obtained from both primiparous and pluriparous cows from animal experiments within a project cooperation, the time course of the mRNA abundance of 12 different target genes and 7 reference genes was characterized in s.c. fat and in liver from pluriparous cows and in three different s.c. and in three v.c. fat depots, in liver, skeletal muscle, and in mammary gland from primiparous cows. Two acute phase proteins, i.e. haptoglobin (Hp) and serum amyloid A3 (*SAA3*), were newly established as adipokines in cattle; both mRNAs yielded similar time course patterns with a peripartal peak. Treatment with CLA was mostly not affecting Hp and *SAA3* mRNA expression; the decrease observed for Hp and *SAA3* mRNA in 2 out of 6 fat depots tested indicates local anti-inflammatory effects of CLA. No CLA effect was observed for the Hp serum concentrations and for hepatic Hp mRNA. Indeed, we confirmed liver as the main site of Hp production. For the prioritization of nutrient uptake towards the mammary gland, IS in other peripheral organs is knowingly reduced. The mRNA expression of the target genes related with IS, i.e. adiponectin (*ADIPOQ*), leptin (*LEP)*, their receptors (*LEPR*, *LEPRB*, *ADIPOR1*, *ADIPOR2*), of two nuclear receptor isoforms (*PPARγ*, *PPARγ2*) and of two pro-inflammatory cytokines (*TNF-α*, *IL-6*) in s.c.AT and in liver from pluriparous cows was mostly decreased from day 21 prepartum to day 21 postpartum in s.c.AT except *TNF-α*; in liver increases were observed for *LEPRB* and *ADIPOR2*, and decreasing abundance for all other hepatic target mRNAs except *TNF-α* and *ADIPOR1* which remained constant in this time. In later lactation, prepartum values were reached again and were largely maintained until wk 36. The groups treated with CLA or control fat differed detachedly in mRNA abundance of *PPARγ*, *LEPRB* and *TNF-α* in liver and of *PPARγ2* in s.c.AT; cows of the CLA group had also higher insulin concentrations and reduced systemic IS persisting after the end of CLA supplementation. In primiparous cows, changes with the duration of lactation were observed for most of the target mRNAs (except *LEP*) but not in all tissues investigated; time course and direction of change were partly divergent between the different tissues. CLA treatment for 105 days decreased the mRNA abundance of *ADIPOQ*, *ADIPOR2*, *PPARγ2* and *TNF-α* in v.c.AT and in the mammary gland. The results of these studies provide a longitudinal characterization of the expression of genes that are particularly related to AT as a heterogeneous functional regulator in lactating dairy cows. The known effect of CLA inhibiting milk fat synthesis might at least be partly explained by the down-regulation of *PPARγ2* in the mammary gland observed herein. The importance of the CLA induced effects on IS for animal health can presently not be finally assessed due to lack of validated reference values for IS in high yielding dairy cows.

German abstract

Zur Kompensation des Energieverlustes über die Milch zu Beginn der Laktation müssen Körperreserven, vor allem Fett, mobilisiert werden. Diese metabolische Anpassung wird durch verschiedene Hormone reguliert. Im Gegensatz zu den „klassischen Hormonen“ die Geburt, Laktation und Stoffwechsel kontrollieren, ist die Bedeutung spezieller Signalmoleküle des Fettgewebes, den sogenannten Adipokinen, sowie deren Rezeptoren und verschiedener Kernrezeptoren bei Milchkühen bisher kaum untersucht. Insbesondere Daten zum Fettgewebe waren bislang primär auf das für Biopsien leicht zugängliche subkutane Fettgewebe limitiert, wobei die Lokalisation am Schwanzansatz im Fokus stand. Mögliche Unterschiede zwischen verschiedenen subkutanen Depots sowie zu den einzelnen viszeralen Depots waren dagegen nicht herausgearbeitet worden. Ziel der vorliegenden Dissertation war daher die Charakterisierung der mRNA-Expression verschiedener Adipokine sowie verwandter Faktoren, welche einen Einfluss auf Insulinsensitivität (IS) und Entzündungsprozesse im Übergang von der späten Gravidität zur Laktation und danach während der Laktation haben. Zusätzlich sollte der mögliche Einfluss konjugierter Linolsäuren (CLA) oder eines Kontrollfettes (Supplementation von Tag 1 bis 105 bzw. 185 der Laktation) auf die Zielgene ermittelt werden. Das Hauptaugenmerk lag hierbei auf dem Fettgewebe (AT) unterschiedlicher Lokalisationen. Als Voraussetzung für die unternommenen Studien erfolgte zu Beginn die Etablierung geeigneter Referenzgene. Bei primi- und pluriparen Kühen wurde der zeitliche Verlauf der mRNA-Expression von zwölf Ziel- und sieben Referenzgenen charakterisiert. Bei pluriparen Tieren erfolgte die mRNA-Quantifizierung im subkutanen Fettdepot am Schwanzansatz sowie in der Leber; bei primiparen Kühen zusätzlich in zwei weiteren subkutanen sowie drei viszeralen Fettdepots, in der Skelettmuskulatur sowie in der Milchdrüse. Zwei Akute Phase Proteine (APP), Haptoglobin (Hp) und Serum Amyloid A3 (*SAA3*), wurden als neue Adipokine des Rindes identifiziert. Die mRNAs beider APP wiesen den gleichen Zeitverlauf mit einem Peak um den Geburtszeitraum auf; CLA beeinflusste weder die Hp- noch die *SAA3* mRNA-Expression. Die sinkende Expression in zwei der sechs untersuchten Fettdepots deutet auf lokale anti-inflammatorische CLA-Effekte. CLA beeinflusste dabei weder die Hp-Serumkonzentrationen noch die mRNA-Expression in der Leber. Die vorliegenden Ergebnisse bestätigten zudem die Leber als den Hauptort der Hp-Synthese. Um die Nährstoffaufnahme der Milchdrüse zu priorisieren, sinkt die IS der übrigen peripheren Organe. Die mRNA-Expression von mit IS in Bezug stehenden Zielgenen Adiponektin (*ADIPOQ*), Leptin (*LEP*), deren Rezeptoren (*LEPR*, *LEPRB*, *ADIPOR1*, *ADIPOR2*), zweier Kernrezeptorisoformen (*PPARγ*, *PPARγ2*), sowie zweier pro-inflammatorischer Zytokine (*TNF-α*, *IL-6*) zeigte mit Ausnahme von *TNF-α* im s.c.AT bei pluriparen Kühen eine Verringerung zwischen Tag 21 vor der Geburt des Kalbes und Tag 21 danach. In der Leber waren *LEPRB* und *ADIPOR1* erhöht, während die übrigen Zielgene, ausgenommen *TNF-α* und *ADIPOR1*, welche konstant exprimiert waren, an Tag 21 postpartum im Vergleich zu Tag 21 antepartum eine verringerte mRNA-Expression aufwiesen. In der späten Laktation erreichten die Werte wieder das vorgeburtliche Niveau und blieben konstant bis Woche 36. Für die mRNA-Expressionen von *PPARγ*, *LEPRB* und *TNF-α* in der Leber und *PPARγ* im s.c.AT zeigten sich fütterungsbedingte Unterschiede. Die Versuchstiere der CLA-Gruppe wiesen zudem höhere Insulinkonzentrationen sowie eine reduzierte systemische IS auf, die über das Ende der CLA-Supplementation Bestand hatten. Bei primiparen Kühen zeigten sich für die meisten Zielgene (ausgenommen *LEP*) laktationsbedingte Veränderungen. Diese konnten jedoch nicht in allen Geweben nachgewiesen werden. Der zeitliche Verlauf sowie die Änderungsrichtung wiesen leichte Unterschiede zwischen den Geweben auf. Die CLA-Supplementation über 105 Tage verringerte die mRNA-Expression von *ADIPOQ*, *ADIPOR2*, *PPARγ* und *TNF-α* in v.c.AT sowie in der Milchdrüse. Die Ergebnisse der vorliegenden Arbeit stellen eine longitudinale Charakterisierung der Expression von Genen zur Verfügung, die bei Milchkühen in Zusammenhang mit AT als funktionellem Regulationsorgan stehen. Der hemmende CLA-Effekt auf die Milchfettsynthese kann zumindest teilweise durch die hier ermittelte Down-Regulation von *PPARγ* in der Milchdrüse erklärt werden. Die Bedeutung der hier beobachteten, CLA-induzierten Effekte auf die IS in Hinblick auf die Tiergesundheit kann derzeit aufgrund fehlender Referenzwerte für die IS bei hochleistenden Milchkühen noch nicht endgültig bewertet werden.

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List of abbreviations

ACC	acetyl-CoA carboxylase
APP	acute phase protein
APR	acute phase response
A-SAA	acute phase SAA
AGPAT	acyl glycerol phosphate acyl transfrase
ADIPOQ	adiponectin
ADIPOR1 and ADIPOR2	adiponectin receptor 1 and 2
AT	adipose tissue
AGP	alpha-1-acid glycoprotein
a.p.	antepartum
BHB or BHBA	beta-hydroxybutyrate
BCS	body condition score
c9,t11-CLA	*cis*-9, *trans*-11 isomer
CoA	coenzyme A
CLA	conjugated linoleic acids
C-SAA	constitutively SAA
CTR	control group
CPT-1	carnitine palmitoyltransferase-1
FASN	de novo fatty acid synthesis
DM	dry matter
EIF3K	eukaryotic translation initiation factor 3, subunit K
EMD	emerin
Epi	epinephrine
M	expression stability
FABP	fatty acid binding protein
FAS	fatty acid synthase
DAAD	German Academic Exchange Service
DFG	German Research Foundation
GfE	German Society of Nutrition Physiology
GPAT	glycerol-3 phosphate acyl transfrase
Hp	haptoglobin

HDL	high density lipoprotein fraction
HRP	horseradish peroxidase
HPCAL1	hippocalcin-like 1
IPA	ingenuity pathway analysis
INSIG1	insulin induced gene 1
IS	insulin sensitivity
IL-6	interleukin-6
JAK	janus kinase
LEP	leptin
LEPR or ObR	leptin receptor
LEPRB or ObRb	leptin receptor isoform b
C18:2, n-6	linoleic acid
LPS	lipopolysaccharide
LPL	lipoprotein lipase
LCFA	long chain fatty acids
LRP10	lipoprotein receptor-related protein 10
M-SAA3	mammary SAA3
MARVELD1	marvel domain containing 1
MFD	milk fat depression
NE	norepinephrine
NEB	negative energy balance
NEFA	nonesterified fatty acids
V	pairwise variation
PMR	partial mixed ration
PPRE	peroxisome proliferator response elements
PPARγ	peroxisome proliferator-activated receptor-γ
POLR2A	RNA polymerase II
PVDF	polyvinyl difluoride membranes
p.p.	postpartum
PPAR	peroxisome proliferator-activated receptor
PPARγ2	peroxisome proliferator-activated receptor-γ2
REF	reference genes
RXR	retinoid X receptor

RQUICKI	revised quantitative insulin sensitivity check index
Roth	Roti®-Histol
SAA	serum amyloid A
SAA3	serum amyloid A3
STAT	signal transducers and activators of transcription
SCD	stearoyl-CoA desaturase
SREBP1	sterol regulatory element binding transcription factor 1
s.c.	subcutaneous
TG	triglyceride
THRSP or S14	thyroid hormone responsive spot 14
t10,c12-CLA	*trans*-10, *cis*-12 isomer
TNF-α	tumor necrosis factor-α
TZD	thiazolidinediones
v.c.	visceral
VLDL	very low density lipoprotein

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leptin receptor isoform b (*LEPRB*), and tumor necrosis factor (*TNF*)-α in liver and s.c. tail head adipose tissue biopsies of dairy pluriparous cows fed with conjugated linoleic acids (CLA, Lutrell® Pure, BASF SE, Ludwigshafen, Germany) at 100 g/day CLA or a control fat supplement (Silafat®, BASF SE) from day 1 until day 182 postpartum in trial 1

Figure 4 Glucose and insulin concentration in serum and calculated Revised Quantitative Insulin Sensitivity Check Index (RQUICKI) in cows receiving conjugated linoleic acids (CLA, Lutrell® Pure, BASF SE, Ludwigshafen, Germany) at 100 g/day or a control fat supplement (Silafat®, BASF SE) from day 1 until day 182 postpartum in trial 1

Figure 5 Individual fat depot differences of adiponectin (*ADIPOQ*), adiponectin receptors (*ADIPOR1* and *ADIPOR2*), leptin (*LEP*), leptin receptor isoform b (*LEPRB*), peroxisome proliferator-activated receptor (*PPAR*) γ and $\gamma 2$, tumor necrosis factor (*TNF*)-α, and interleukin (*IL*)-*6* mRNA abundance [mean of d 1 (n = 5), 42 (n = 10), and 105 (n = 10) postpartum from primiparous cows (trial 2) fed at 100 g/day with conjugated linoleic acids (CLA, Lutrell® Pure, BASF SE, Ludwigshafen, Germany) or a control fat supplement (Silafat®, BASF SE)] in trial 2

1. Introduction

With livestock animals as matter of research, physiology needs to consider a complex system with numerous interactions and feedback mechanisms. This system does not only comprise the biological organism of the animal itself, but also the herd with the interactions between animals, and the underlying technical and human subsystems involving the natural environment and also socio-economic factors. In view of the worldwide declining arable land and increasing population, the use of land for "food, feed or fuel" needs to be balanced. The ability of livestock, in particular ruminants, to make use of grasslands otherwise not accessible for food production, as well as the option of using human food "waste" for feed provides an opportunity to feed more people and also presents other advantages.

With this background, animals that are able to efficiently convert nutrients into milk or meat are required. Genetic selection for increased productivity can have negative side effects on animal health and welfare. For example, in dairy cows increasing milk yield is commonly related to metabolic disorders, to reduced fertility, lameness and compromised immune function and thus increased susceptibility towards infectious disease e.g. mastitis. In consequence, the turnover rates within a herd increase resulting in a shorter productive life of the animals. To reduce these negative effects different managerial, nutritional, and genetic approaches are used. Nowadays, different feed supplements are introduced aiming to improve health of farm animals and thus counteracting the short productive life.

1.1. Negative energy balance and insulin sensitivity during early lactation

During early lactation, dairy cows undergo a state of negative energy balance (**NEB**) and cows have to mobilize body resources, mainly body fat, to cover the output of energy via milk. Voluntary feed intake does not increase as fast as milk production increases in early lactation; in adaptation to this, homeorhetic changes of hormonal regulation and, in consequence, metabolism occur, leading to a repartitioning of nutrients mainly from fat reserves to the mammary gland (Bell and Bauman, 1997).

Glucose is a primary fuel for fetal life. The placenta translocates glucose from maternal blood circulation in an insulin-independent manner (Bell and Bauman, 1997). This process is facilitated by the development of insulin resistance in peripheral tissues. In

early lactation, the peripheral insulin sensitivity is further reduced, e.g. in muscle and adipose tissue (**AT**) allowing to direct glucose towards the mammary gland (Bell, 1995). Similar to placenta, glucose uptake is an insulin-independent process in the mammary gland tissue as well. The endocrine mechanisms affecting insulin sensitivity are well-documented but the role of AT derived messenger molecules, i.e. of adipokines and also of their receptors in these adaptive processes are only at the outset of being known and understood.

1.1.1. Homeorhetic adaptations around parturition

The importance of a successful transition from pregnancy to early lactation is well accepted and determines the profitability of dairy cows. Health problems, nutrient deficiency, or poor management may impede the ability of cows to reach maximal (production) efficiency (Drackley, 1999). The most critical time for dairy cows is early lactation during which the energy requirements increase up to 4 fold in a short time from pregnancy and dry period to lactation (Carriquiry et al., 2009). In compensation of the new requirements, cows start to mobilize body fat and muscle tissue. Increasing growth hormone serum concentrations directly after parturition (Gross et al., 2011; Doepel et al., 2002) force liver to increase gluconeogenesis and AT to reduce lipogenesis (Renaville et al., 2002). Growth hormone increases the utilization of nonesterified fatty acids (**NEFA**) indirectly via IGF-1 in muscle and in mammary gland by increased blood flow as reviewed by Renaville et al. (2002). Most of the energy requirement during lactation is needed for milk production i.e. in 4 days after parturition, 97% of net energy and 83% of metabolizable energy based on energy and protein intake is used for milk production (Drackley, 1999). The nutrient uptake of the mammary gland is insulin independent therefore other insulin dependent tissues start to increase fatty acid oxidation and to decrease glucose uptake and utilization because of the low plasma insulin concentrations and the decrease in insulin sensitivity in early lactation (Butler et al., 2003). Liefers et al. (2003) showed that the leptin (***LEP***) concentration is high in the dry period and declines to a nadir at parturition thus they suggest that, at least in short term, *LEP* reflects energy balance consequently it is low during early lactation. Recent studies on obesity summarized by Ahima and Osei (2008) identified many new molecules related to insulin sensitivity i.e. adiponectin (***ADIPOQ***) and its receptors (***ADIPOR1*** and ***ADIPOR2***), *LEP* and its receptors [e.g. *LEP* receptor isoform b (***LEPRB***)], interleukin-6 (***IL-6***), tumor necrosis factor-α (***TNF-α***), peroxisome proliferator-activated receptor-γ (***PPARγ***),

PPARγ2, etc. Many of these molecules are AT derived and are named adipokines. Although different aspects of these molecules are well established in monogastrics, there is less information available in ruminants.

1.1.2. Lipid metabolism during the transition period

As already mentioned in previous sections, early lactation is accompanied with NEB and reduced insulin sensitivity. In normal conditions when tissues are insulin responsive, AT deposits fatty acids in the form of triglycerides. Under reduced insulin sensitivity conditions or low insulin concentrations in serum, a situation typical for early lactation, AT starts lipolysis and thus serum NEFA concentrations increase. Extreme rates of lipolysis in AT increase the uptake of NEFA by liver and the liver triglyceride content (Figure 1). In early lactation, cows have a high total lipid and triacylglycerol content in liver whereas glycogen content decreases (Drackley et al., 2005). In severe cases hepatic lipidosis or fatty liver may happen. Otherwise, NEFA is metabolized to ketone bodies or

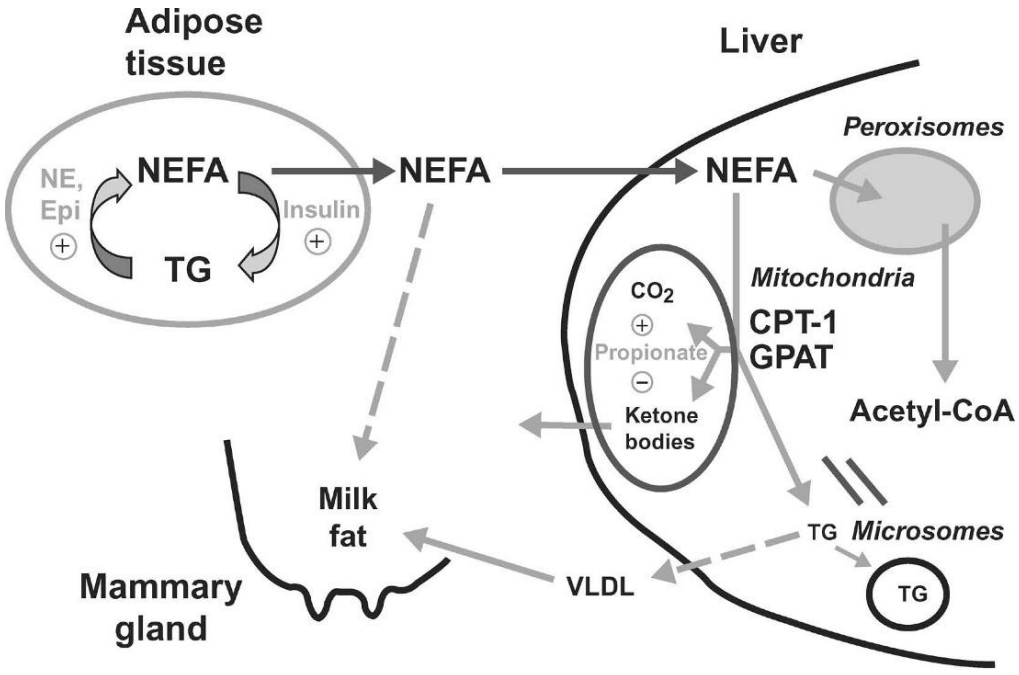

Figure 1: Schematic of metabolic relationships between AT, liver, and mammary gland during the transition period; NE = norepinephrine, Epi = epinephrine, CPT-1 = carnitine palmitoyltransferase-1, GPAT = glycerol-3-phosphate acyltransferase, TG = triglyceride, CoA = coenzyme A, VLDL = very low density lipoprotein (adapted from Drackley et al., 2006).

is released from liver in the form of very low density lipoproteins (**VLDL**). Both NEFA and VLDL are also used in milk fat production as well (Drackley, 1999; Drackley et al., 2006).

1.2. Regulators of insulin sensitivity

1.2.1. *ADIPOQ* system

ADIPOQ was identified in 2001 in bovine fetal serum as a glycoprotein. *ADIPOQ* shows 92 and 82% identity with murine and human *ADIPOQ*, respectively (Sato et al., 2001). *ADIPOQ* is secreted mainly from AT and exerts insulin sensitizing effects via adiponectin receptor 1 and 2 (***ADIPOR1/2***; Figure 2) in humans (Kadowaki and Yamauchi, 2005). The *ADIPOR1* is ubiquitously expressed, including abundant expression in skeletal muscle. The *ADIPOR2* is most abundantly expressed in liver (Kadowaki et al., 2006). *ADIPOQ* reduces the tissue triglyceride content in muscle and liver thus increasing insulin sensitivity via increasing the expression of *PPARα*. *ADIPOQ* also activates AMP activated protein kinase thereby stimulating ß-oxidation and thus decreasing the triglyceride content in liver and in muscle tissue (Kadowaki and Yamauchi, 2005).

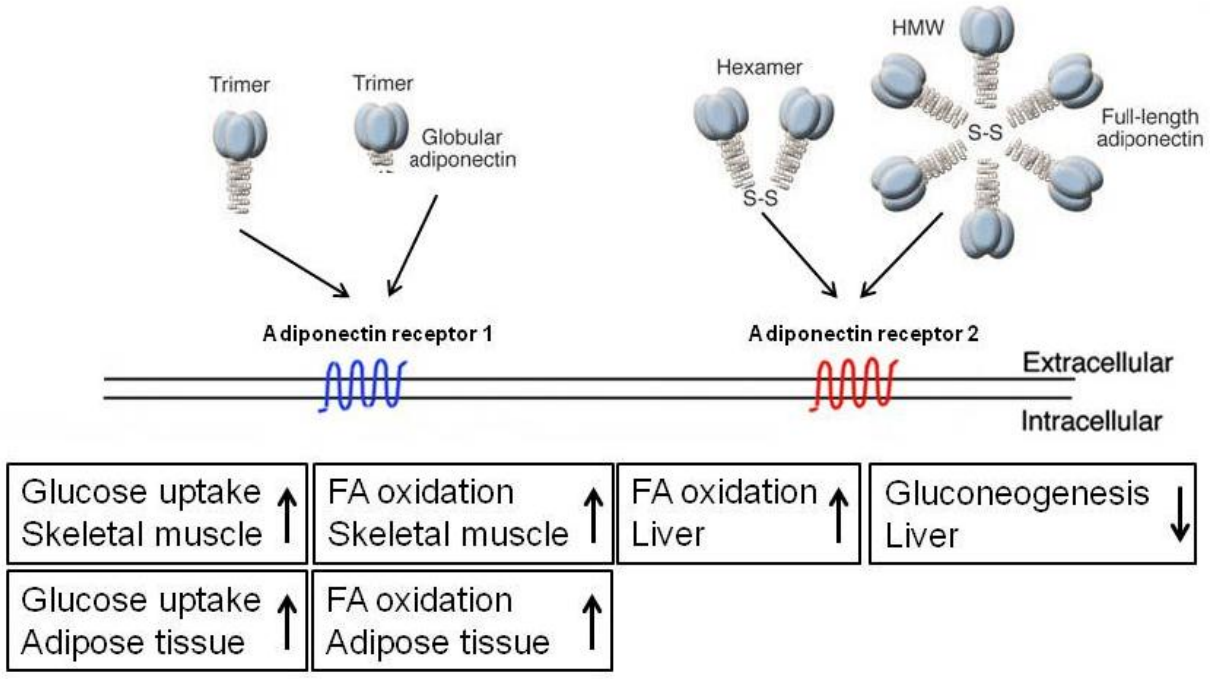

Figure 2: Adiponectin action and its receptors and their functions in different tissues (revised after Kadowaki and Yamauchi, 2005 using Wu et al., 2003 and Puigserver et al., 2001).

In dairy cattle, the mRNA abundance of *ADIPOQ* in subcutaneous (**s.c.**) AT has been reported to be constant (Lemor et al., 2009) or to decrease (Koltes and Spurlock, 2012) during the transition from pregnancy to lactation. In intestinal AT, a down-regulation of *ADIPOQ* mRNA from the dry period to peak and late lactation was demonstrated (Komatsu et al., 2007). For *ADIPOR1* and *ADIPOR2* in s.c.AT, constant (Sadri et al., 2010b) or decreasing values were reported (Lemor et al., 2009). Reduced abundance of *ADIPOQ* and its receptors in AT point to reduced insulin sensitivity during early lactation. *ADIPOQ* is negatively correlated with fat mass and adipocyte size, respectively (Cnop et al., 2003; Skurk et al., 2007).

Studying mammary tissue samples, Ohtani et al. (2011) showed a stable expression of *ADIPOR1* and *ADIPOR2* in peak [60 day postpartum (**p.p.**)] and late (240 day p.p.) lactation. Indeed, *ADIPOR2* had the same level of expression in the dry period in comparison to the lactation period whereas *ADIPOR1* was down-regulated.

1.2.2. *LEP* system

LEP was first identified in mice (Zhang et al., 1994) and subsequently in sheep (Dyer et al., 1997) and cattle (Ji et al., 1998). *LEP* is highly conserved across all species, with human *LEP* showing 84 and 83% homology with mouse and rat *LEP* (Zhang et al., 1994). The word *LEP* comes from the Greek word *Leptos* which means thin. *LEP* regulates feed intake via its effects on the central nervous system. Increasing *LEP* suppresses feed intake and increases energy expenditure (Ricci and Bevilacqua, 2012).

LEP is the first adipokine identified and is secreted mainly but not exclusively from AT and its expression is proportional to fat mass and adipocyte size (Delavaud et al., 2002). *LEP* affects energy homeostasis and improves insulin sensitivity (Houseknecht and Portocarrero, 1998). Serum *LEP* is reduced p.p. vs. antepartum (**a.p.**), but at the level of the mRNA in s.c.AT from tail head of multiparous cows no changes were observed (Lemor et al., 2009; Sadri et al., 2010b). A down-regulation of AT *LEP* mRNA was reported comparing 3 to 10 weeks dry cows with cows in peak lactation, thus AT *LEP* mRNA is higher in dry cows (Komatsu et al., 2007; Thorn et al., 2008).

LEP exerts its effects via its receptors. *LEPR* was first identified in mice (Tartaglia et al., 1995). Multiple splice variants of the *LEPR* gene encode at least 6 different isoforms of *LEPR* in rodents (Ahima and Flier, 2000; Ingvartsen and Boisclair, 2001). They share a common extracellular ligand binding domain but differ in size of the intracellular domain (Figure 3). The long form of *LEPR* - *LEPRB* - has the primary biological role (Bartha et

al., 2005). The *LEPRB* contains intracellular motifs required for activation of the ***JAK*** (janus kinase)-***STAT*** (signal transducers and activators of transcription) signal transduction pathways being the signal transduction pathways attributed to *LEP* (Bjørbæk et al., 1997; Vaisse et al., 1996). The *LEPRB* is mainly expressed in different brain regions, in particular the paraventricular, ventromedial, and arcuate nuclei of the hypothalamus (Williams et al., 1999). *LEPRB* is also expressed in peripheral tissues (Chelikani et al., 2003). In dairy cows' AT, Lemor et al. (2009) reported an increase in mRNA abundance of *LEPRB* comparing days p.p. vs. a.p. consistent with the observations of Thorn et al. (2008) reporting a 10 fold increase in *LEPR* mRNA abundance in AT and a 2 fold increase for the same receptor in liver tissue from late pregnancy to early lactation. Hypoinsulinemia associated to NEB in early lactation might be responsible for the mentioned induction of *LEPRB* in liver and AT (Lemor et al., 2009; Thorn et al., 2008).

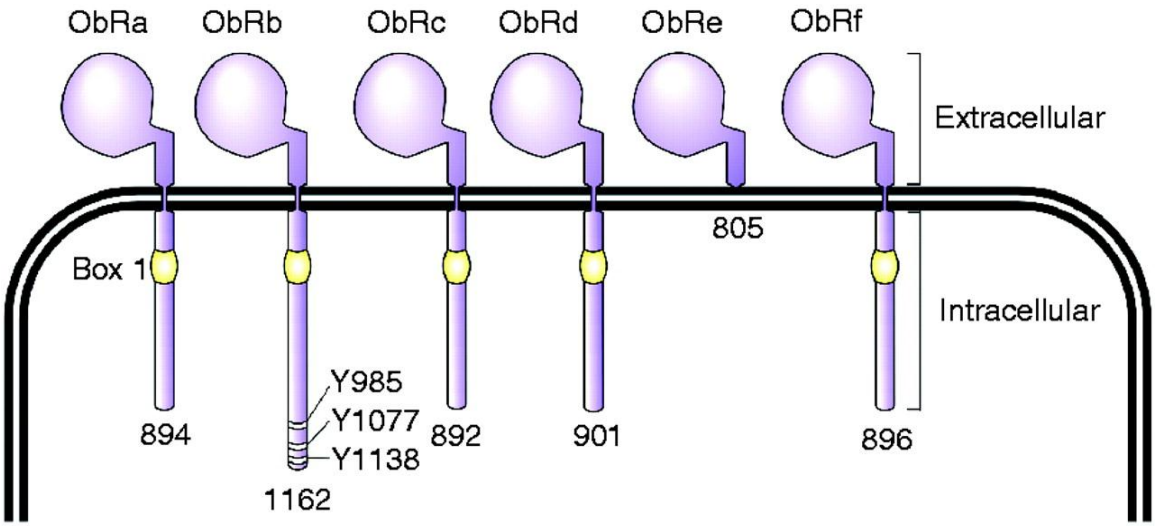

Figure 3: The leptin receptor has six isoforms obtained by alternative splicing, which are designated *ObRa*, *ObRb*, *ObRc*, *ObRd*, *ObRe*, and *ObRf* (also named *LEPRA*, *B*, *C*, *D*, *E*, and *F* respectively). The number below each *LEPR* form indicates the number of amino acids characteristic of each isoform. The box 1 motif is required for *JAK* interaction and activation. However, only the long form (*ObRb*) contains motifs for the complete activation of the JAK/STAT pathway. Three tyrosine residues, whose phosphorylation is important for leptin signaling, are indicated in *LEPRB*: Y985 interacts with the SH2-containing protein tyrosine phosphatase 2, Y1077 with STAT5, and Y1138 with STAT3 (adapted from Marroquí et al., 2012).

1.2.3. *PPARγ* and *PPARγ2*

Peroxisomes are subcellular organelles found in most plant and animal cells that perform diverse metabolic functions including β-oxidation of fatty acids, and cholesterol metabolism. The *PPAR* were identified in 1990 and belong to a nuclear hormone receptor superfamily (Tyagi et al., 2011). Nuclear hormone receptor proteins form a class of ligand activated proteins that, when bound to specific sequences of DNA, serve as on-off switches for transcription within the cell nucleus (Abbott, 2009). The *PPAR* regulate gene expression via binding to specific DNA sequences, peroxisome proliferator response elements (***PPRE***), in the promoter regions of target genes (Figure 4). Prior to DNA binding, *PPAR* forms a heterodimer with the retinoid X receptor (***RXR***), another member of the nuclear receptor super family (Abbott, 2009).

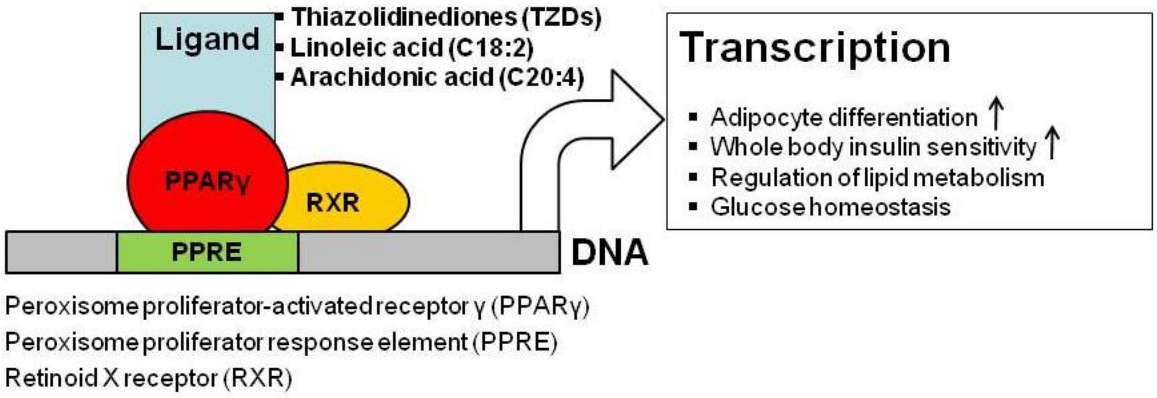

Figure 4: Signal transduction of genes regulated by peroxisome proliferator-activated receptor γ (revised after Houseknecht et al., 2002 using Rosen and MacDougald, 2006 and Herrmann et al., 2009).

The family of *PPAR* includes three members (Figure 5): *PPARα*, *PPARδ*, and *PPARγ*. They play an essential role in energy metabolism; however, they differ in the spectrum of their activity. *PPARγ* regulates energy storage in AT, whereas *PPARα* is expressed predominantly in the liver, and to a lesser extent, in muscle, in the heart, and in bone. *PPARδ*, ubiquitously expressed in the whole body, regulates energy expenditure. *PPARγ* is further subdivided in four isoforms (Evans et al., 2004):

- γ1 - expressed in virtually all tissues, including heart, muscle, colon, kidney, pancreas, and spleen.

- γ2 - expressed mainly in AT.
- γ3 - expressed in macrophages, large intestine, and AT.
- γ4 - expressed in endothelial cells.

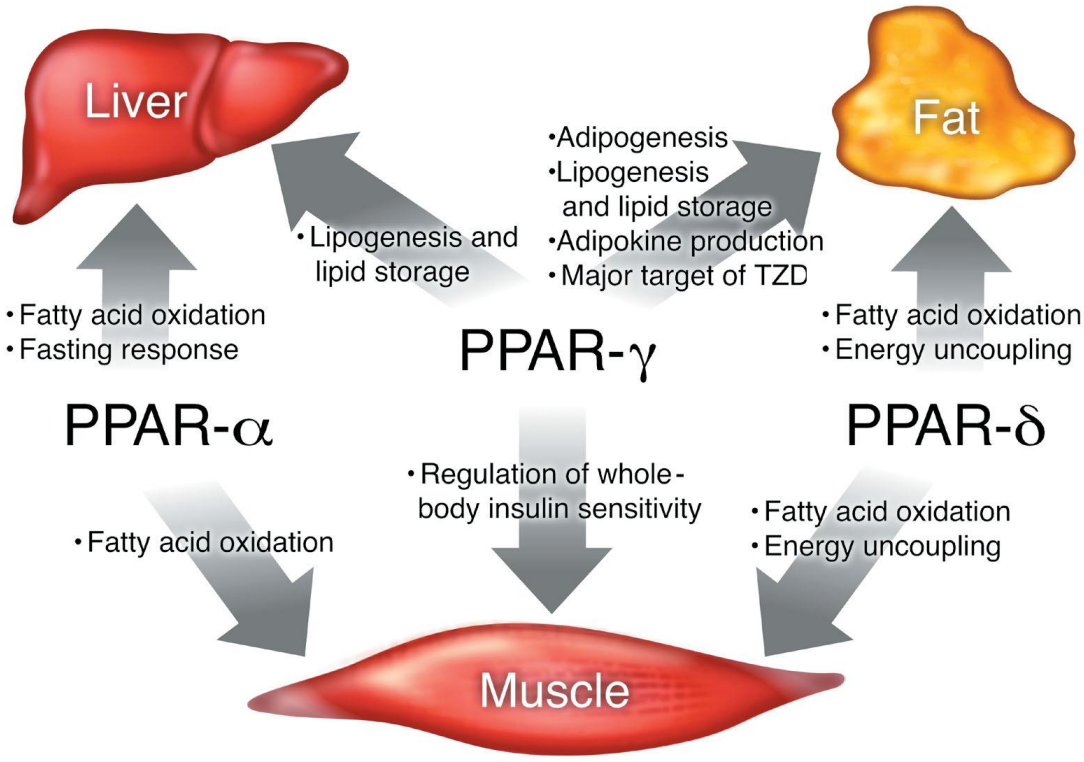

Figure 5: Metabolic integration of *PPAR* regulating energy homeostasis in liver, muscle, and adipose tissue (adapted from Evans et al., 2004). TZD: Thiazolidinediones, *PPAR*: peroxisome proliferator-activated receptor.

PPARγ2 is a nuclear receptor highly expressed in AT (Herrmann et al., 2009). It is important for adipocyte differentiation and regulates insulin sensitivity by transcriptionally activating adipocyte-specific genes involved in insulin signaling, glucose and fatty acid uptake, and lipid-storage (Herrmann et al., 2009). There are different agonists for *PPARγ*. Linoleic and arachidonic acid are two natural agonists. Thiazolidinediones (**TZD**) e.g. Ciglitazone, Pioglitazone, and Rosiglitazone are synthetic agonists (Nosjean and Boutin, 2002). *PPARγ2* agonists increase plasma *ADIPOQ* concentrations and thus improve whole body insulin sensitivity in monogastrics (Combs et al., 2004).

1.2.3.1. *PPARγ2* in AT

The peripartal plasticity of AT in response to changes in hormones and specific nutrients

is well documented and is under control of regulators of transcription like *PPARγ* (Loor, 2010). *PPARγ* is a key regulator of insulin sensitivity in non-ruminants (Rosen and MacDougald, 2006). Therefore, nutritional regulation of *PPARγ* around parturition might support insulin responsiveness in bovine AT. The *PPARγ* mRNA is stably expressed after parturition up to 3 weeks p.p. in AT of dairy cows without differences between cows with high or low lipolytic activity as indicated by plasma NEFA concentrations (van Dorland et al., 2011). In addition, no difference between *PPARγ* mRNA expression in AT of cows in peak and late lactation was observed as well (Komatsu et al., 2007). *PPARγ* mRNA abundance in AT increases in cows fed with a high energy diet in comparison to low energy diet. In the same experiment, they found no effect of TZD administration to diet on AT *PPARγ* mRNA (Schoenberg and Overton, 2011). For the first time in dairy cattle, Schoenberg et al. (2011b) reported that TZD up-regulate expression of *PPARγ* mRNA in s.c. tail head fat as well as *LEP* mRNA similar to monogastrics. Therefore, *PPARγ* has an important regulatory effect in bovine AT similar to monogastrics.

1.2.3.2. *PPARγ2* in the mammary gland

In bovine mammary tissue, *PPARγ2* is probably localized in epithelial cells (Bionaz and Loor, 2008). Bionaz and Loor (2008) supported this hypothesis by the low expression of adipocyte specific genes indicating a low number of adipocytes in lactating mammary parenchyma. *PPARγ2* is up-regulated up to 3 fold at the onset and throughout 240 days in lactation in comparison to 15 d a.p. Previously, similar data was reported for *PPARγ*. Although lactating cows were compared to cows dried-off for 3 to 10 weeks a.p., *PPARγ* was stably expressed during the dry period and peak and late lactation (Komatsu et al., 2007). *PPARγ2* agonists i.e. rosiglitazone and long chain fatty acids were not directly influencing mammary *PPARγ2* mRNA expression. However, *PPARγ2* putative target genes (mostly genes related to lipid synthesis) were up-regulated by treatment with *PPARγ2* agonists (Kadegowda et al., 2009).

1.2.3.3. *PPARγ2* in liver

Liver has a major role in energy metabolism during the transition period. Studies in monogastrics associated liver *PPARγ* with triglyceride homeostasis (Gavrilova et al., 2003) as reviewed by Rogue et al. (2010). Deletion of *PPARγ* in liver causes impairment in insulin sensitivity (Gavrilova et al., 2003; Matsusue et al., 2003). A decrease in the triglyceride content of the liver, a reduction in mRNA abundance of genes dealing with

lipid metabolism, and an increase in triglycerides and free fatty acids content occurs in plasma of mice with *PPARγ* gene deficiency. In addition, *PPARγ2* but not *PPARγ1* in murine liver was up-regulated in response to a high butter fat diet (Yamazaki et al., 2011) Thus, high fat diets will induce fatty liver in a *PPARγ2* dependent manner.

In dairy cows, feeding a mixture of 50% each of the *cis*-9, *trans*-11 and the *trans*-10, *cis*-12 isomer of conjugated linoleic acid (**CLA**) to dairy cows during late pregnancy and early lactation had no effect on *PPARγ* expression in liver tissue biopsies (Sigl et al., 2010). This observation regarding to changes in *PPARγ* mRNA a.p. and p.p. based on the real-time PCR quantification cycle values revealed no changes in this time period. In an experiment where cows were grouped based on plasma *beta-hydroxybutyrate* (**BHB**) concentrations (van Dorland et al., 2009), no difference in liver *PPARγ* mRNA was found by BHB grouping or by days around parturition (day 70 a.p. to 98 p.p.). In another experiment, hepatic gene expression profiling between cows in mild NEB and severe NEB revealed no shift in *PPARγ* mRNA abundance (McCarthy et al., 2010).

1.2.3.4. *PPARγ2* in muscle and pancreas

In a murine muscle cell line, *PPARγ2* follows the same regulatory pathways as in adipocytes, i.e. *PPARγ2* is reduced in inflammation (*TNF-α* treatment) and the omega-3 polyunsaturated fatty acid eicosapentaenoic acid attenuates this effect (Magee et al., 2012). Indeed, muscle *PPARγ* mRNA abundance increased more than 10 fold in growing steers from 10 to 22 months of age when the size of intramuscular adipocytes was increasing independently of breed (Albrecht et al., 2011). A very low abundance of *PPARγ2* in muscle tissue in comparison to AT was demonstrated as well (Huff et al., 2004).

The *PPARγ* is expressed in pancreatic ß-cells and it could be related to lipotoxic effects of fatty acids in these cells (Kawai et al., 2002). Overexpression of *PPARγ* suppresses insulin secretory capacity of pancreatic islets (Ito et al., 2004). Moreover, *PPARγ* inhibits glucagon gene transcription in islets of pancreatic tissues (Krätzner et al., 2008). Nevertheless, it remains controversial whether *PPARγ* contributes to the function of ß-cells (Welters et al., 2004).

1.2.3.5. Conflicting information available about *PPARγ* isoforms

Two isoforms are defined for *PPARγ* gene in cattle i.e. *PPARγ1* (NIH gene back accession number: Y12419) and *PPARγ2* (accession number: Y12420) (Sundvold et al.,

1997). The two isoforms show 90% homology. At the mRNA level, the isoforms can be differentiated only if at least one of the primer pairs is located at the first 259 bp of *PPARγ2* or at the first 155 bp of *PPARγ1* (Figure 6).

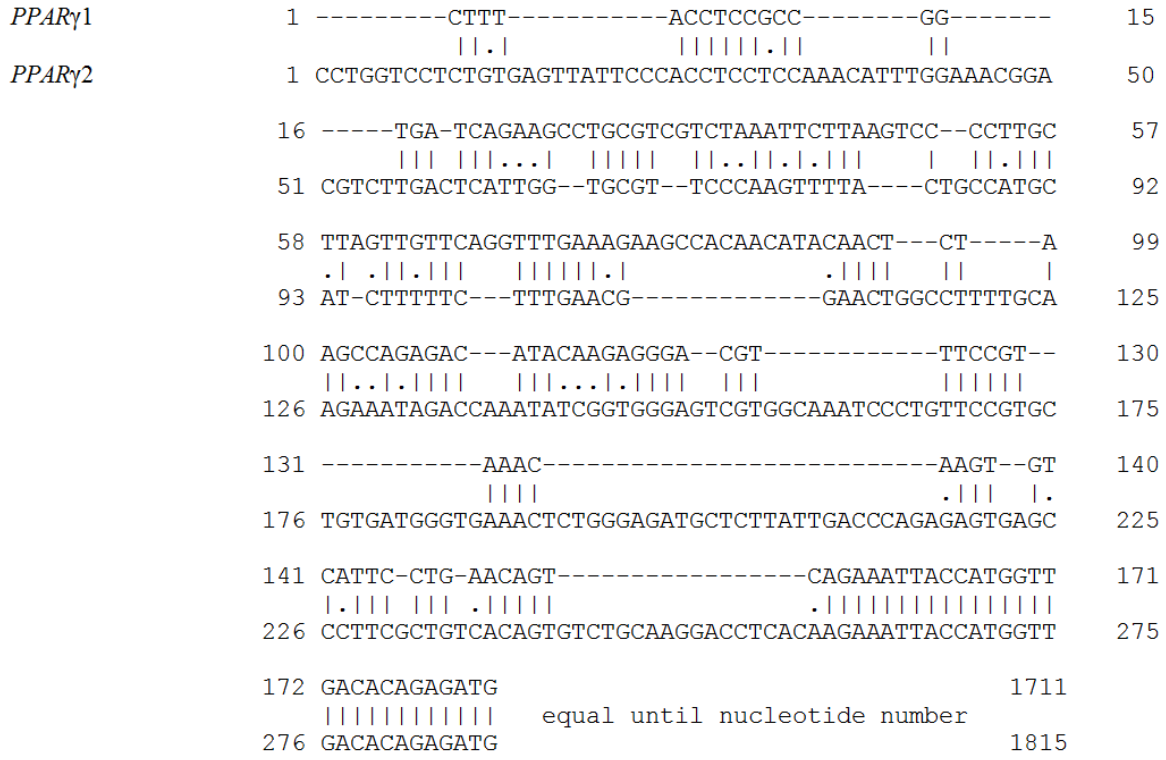

Figure 6. Alignment of bovine peroxisome proliferator-activated receptor γ 1 (*PPARγ1*, NIH gene bank accession number: Y12419) and *PPARγ2* (accession number: Y12420) sequences. Horizontal lines indicate missing base pairs. Vertical lines define homologue sequences. Dots are used for differences between the two sequences. Digits define the number of nucleotides.

Otherwise the primer will not be specific to the isoform. There is another sequence submitted in NCBI database under the name of *PPARγ* (accession number: NM_181024) while is actually 100% identical to *PPARγ2* (accession number: Y12420) and in many cases it is misleading; for example, Kadegowda et al. (2009), Bionaz and Loor, (2008), Schmitt et al. (2011), and Ji et al. (2012) reported a *PPARγ* primer which is actually specific to the *PPARγ2* isoform based on alignment analysis. My survey of the published data (Harvatine et al., 2009; Huff et al., 2004; Komatsu et al., 2007; MacLaren et al., 2006; Mani et al., 2009; McCarthy et al., 2010; Schoenberg and Overton, 2011;

Schoenberg et al., 2011a, b; Sigl et al., 2010; Soliman et al., 2007; van Dorland et al., 2009; van Dorland et al., 2011) revealed that actually many of the published data regarding to *PPARγ* or *PPARγ2* are presenting both isoforms.

1.2.4. *TNF-α* and *IL-6*

TNF-α is a proinflammatory cytokine produced by different cells, mainly by stimulated monocytes. *TNF-α* is involved in inflammation, cell apoptosis, and insulin sensitivity (Antuna-Puente et al., 2008; Galic et al., 2010; Gnacińska et al., 2009; Kershaw and Flier, 2004). High concentrations of *TNF-α* lead to reduced insulin sensitivity (Hotamisligil, 2000) and attenuated secretion of *ADIPOQ* from adipocytes (Maeda et al., 2002). In humans, *TNF-α* is not or only marginally secreted from AT in lean and healthy individuals (Mohamed-Ali et al., 1997). However, *TNF-α* is related to lipid metabolism in AT: the net action of *TNF-α* is to decrease free fatty acid uptake and lipogenesis while increasing lipolysis (Sethi and Hotamisligil, 1999). Indeed, *TNF-α* will increase production of free fatty acids and triglycerides in liver as discussed earlier (Popa et al., 2007).

In dairy cows, a reduction in plasma *TNF-α* is reported after parturition (Schoenberg et al., 2011b; Winkelman et al., 2008). Indeed, recombinant *TNF-α* induced insulin resistance in steers (Kushibiki et al., 2001) and this was also demonstrated in cows with fatty liver (Ohtsuka et al., 2001) indicating negative correlation of serum *TNF-α* with insulin sensitivity.

IL-6, another proinflammatory cytokine, is expressed in different cells i.e. immune cells, endothelial cells, and myocytes. Recently, it was shown that *IL-6* is also expressed in and released from adipocytes. In non-inflammatory conditions, nearby 25% of *IL-6* circulating in blood are estimated to origin from AT in humans (Mohamed-Ali et al., 1997). *IL-6* is closely linked to lipid metabolism (Garcia-Escobar et al., 2010) and insulin resistance (Rotter et al., 2003) beside its role in inflammation. Increased abundance of plasma *IL-6* is linked to greater lipolysis (Morisset et al., 2008). In addition, lipolytic effects of *IL-6* in a porcine adipocyte cell culture system are reported (Yang et al., 2008) similar to human AT (van Hall et al., 2003). Data are still controversial about *IL-6*. In cases of obesity and type 2 diabetes, *IL-6* increases. In contrast, there are studies suggesting beneficial effects of *IL-6* on the disease causing lipolysis without negative effect on insulin-mediated glucose transport (Ji et al., 2011).

1.2.4.1. *IL-6* and *TNF-α* in bovine AT

Both *IL-6* and *TNF-α* are proinflammatory cytokines and initiate an acute phase response (**APR**) in cows (Kushibiki et al., 2003, Nakagawa-Tosa et al., 1995, Yoshioka et al., 2002). However, limited data is available on *IL-6* and *TNF-α* expression in AT of cattle. In dairy cows, AT *TNF-α* mRNA expression is increased from 8 weeks a.p. to the day of parturition and then remains stable for the following 5 weeks p.p. (Sadri et al., 2010a). This links *TNF-α* to the process of body fat mobilization in early lactating cows and initiates a similar role for *TNF-α* in increasing lipolysis in AT as shown for monogastrics (Sethi and Hotamisligil, 1999). In AT culture samples obtained from dairy cows, *IL-6* and *TNF-α* were increased in response to lipopolysacharide (**LPS**) treatment (Mukesh et al., 2010). Thus, adipose depots of dairy cows seems immune responsive and capable of synthesizing proinflammatory cytokines in response to inflammation (Mukesh et al., 2010).

1.2.4.2. *IL-6* and *TNF-α* in bovine liver

In an experiment to test effects of repeated liver biopsies, *IL-6* and *TNF-α* mRNA were detectable in bovine liver biopsies with or without LPS stimulation. The *TNF-α* mRNA abundance was higher than the one of *IL-6* (Vels et al., 2009). Both cytokines were up-regulated after LPS vs. NaCl treatment. In liver of dairy cows, *TNF-α* mRNA exhibited a peak at the day of parturition and another peak at day 49 p.p. (Loor et al., 2005). In addition, restricted energy intake resulted in a more pronounced up-regulation of *TNF-α* (Loor et al., 2006). In consistence with data from monogastrics, treatment of cows with *TNF-α* to introduce chronic inflammation caused an increase in liver triglyceride content thus relating inflammatory pathways to the introduction of fatty liver (Bradford et al., 2009).

IL-6 up-regulates acute phase protein (**APP**) production in hepatocytes isolated from slaughtered cattle if it is added to the cell culture medium in combination with *TNF-α*. However, the presence of only one of these cytokines stimulates concentration of serum amyloid A (***SAA***), but not haptoglobin (Alsemgeest et al., 1996). *IL-6* is expressed in bovine liver Kupffer cells obtained from slaughtered calves and is up-regulated by LPS stimulation (Yoshioka et al., 1998). In microarray and qPCR data of cows with ketosis (Loor et al., 2007), *IL-6* mRNA was 3 fold up-regulated as compared to healthy cows. Network analysis revealed that *IL-6* is related to many liver specific pathways e.g.

lipoprotein metabolism and fatty acid oxidation (Loor et al., 2007). The alteration of pathways affected by *IL-6* might lead to liver lipidosis, ketosis, and insulin resistance (Loor et al., 2007). *IL-6* is thus an important molecule especially regarding the huge amount of NEFA which should be processed in the liver of dairy cows in early lactation.

1.3. Acute phase proteins

Acute phase proteins comprise more than 200 proteins that undergo marked changes in their serum concentration after infection, injury, trauma, or surgery depending on the protein. The APP include negative or positive proteins, i.e. showing a decrease or an increase in their serum concentration in response to stimuli. Albumin and transferrin are examples of negative APP. Haptoglobin, C-reactive protein, *SAA*, ceruloplasmin, fibrinogen, and alpha-1-acid glycoprotein (***AGP***) are examples of positive APP which are mainly produced by hepatocytes although extrahepatic expression was also shown (Murata et al., 2004).

The acute phase response is closely related to the immune response as an early defense mechanism in the body. Production of cytokines and chemokines by immune cells (monocytes, lymphocytes, etc.) is the first step after sensing of antigens or remaining of dead cells by these immune cells. The main proinflammatory cytokines are *IL-6* and *TNF-α*. They have numerous functions as described in the previous chapter including induction of the APR. Production of these cytokines by immune cells causes hepatocytes to produce APP (Cray et al., 2009). The APP have vast biological functions depending on the protein. Herein, the focus will be on haptoglobin and *SAA*.

1.3.1. Haptoglobin

Haptoglobin (**Hp**) is a plasma protein that binds free hemoglobin thereby inhibiting hemoglobin induced oxidative damage (Ceciliani et al., 2012). Haptoglobin comes from the Greek word *haptein* (to bind) and (hemo)globin, and was first discovered in human plasma in the late 1930s. Haptoglobin has different functions as reviewed by Ceciliani et al. (2012) i.e. limitation of iron availability for bacteria, binding hemoglobin and thus prevention of oxidative stress, anti-inflammatory, angiogenesis stimulation, and chaperone-like activity. Haptoglobin in AT might act as a monocyte chemoattractant factor (Maffei et al., 2009).

The Hp and *SAA* are the two main APP in farm animals i.e. cattle, sheep, and goat (Cray et al., 2009). In dairy cows, Hp is the most prominent APP. Although *SAA* response is

quicker than Hp (Heegaard et al., 2000), the Hp concentrations in plasma increase more in comparison to *SAA* in dairy cows (Stenfeldt et al., 2011). Measurement of APP in blood is used as diagnostic marker for diseases as summarized elsewhere (Ceciliani et al., 2012; Cray et al., 2009).

Haptoglobin was shown to be expressed in different cells i.e. leukocytes and milk somatic cells (Thielen et al., 2005) and tissues i.e. liver (Yoshioka et al., 2002), reproductive system (Lavery et al., 2004), digestive tract (Dilda et al., 2012), and mammary gland (Lai et al., 2009; Thielen et al., 2007). Liver is the main source of Hp (Lecchi et al., 2009). However, Hp expression in bovine AT has not been shown until now.

The Hp concentration in blood from healthy dairy cattle is low (< 20 μg/mL). In inflammatory situations, the Hp concentration in blood increases. For example, during an experimental model of dairy cow mastitis (Eckersall et al., 2006), serum Hp concentration increased 5 fold in comparison to a saline control. Injection of 2.5 μg/kg body weight per day recombinant *TNF-α* to lactating dairy cows (Kushibiki et al., 2003) increased serum concentrations of Hp more than 1000 fold in comparison to a saline control. Indeed, APP are related to the mild inflammatory conditions related to obesity and the consequent insulin resistance in humans (Festa et al., 2002). In dairy cows, the association between Hp concentrations in serum and in milk and the metabolic status of dairy cows peripartal was demonstrated (Hiss et al., 2009) i.e. cows having higher NEFA and BHB exhibiting higher Hp in serum.

1.3.2. Serum amyloid A

SAA is an APP belonging to a family of apolipoproteins. In cattle, pigs, horses, dogs, and cats, *SAA* is a major APP (Eckersal and Bell, 2010). In 1988, the *SAA* protein was characterized in dairy cows (Husebekk et al., 1988). Expression of *SAA* increases up to 1000 fold in response to various injuries, infection, etc. From an evolutionary point of view, *SAA* is a highly conserved protein detectable in different vertebrates. Four different *SAA* isoforms have been detected in humans and in mice. Two acute phase *SAA* (***A-SAA***) i.e. *SAA1* and *SAA2* are mainly expressed in liver (Uhlar and Whitehead, 1999). Other isoforms are *SAA3* and *SAA4*. *SAA3* is mainly expressed extrahepatically in rat and mice (Chiba et al., 2009; McDonald et al., 2001). Previously, *SAA3* was known as a pseudogene in human. In 2003, McDonald's group characterized a mammary *SAA3* (***M-SAA3***) in humans that was slightly different in size in comparison to other mammals (Larson et al., 2003). *SAA3* was shown to be secreted from mammary epithelial cells into

colostrum and milk (Molenaar et al., 2009; Eckersall et al., 2006). *SAA3* is the predominant *SAA* isoform in AT (Scheja et al., 2008). *SAA4* is a constitutively expressed *SAA* isoform (***C-SAA4***) which is moderately expressed in response to inflammatory stimuli (de Beer et al., 1996).

The biological functions of *SAA* are poorly understood. However, *SAA* binds to cholesterol, modulates the innate immune system, and opsonizes gram positive and negative bacteria (Ceciliani et al., 2012). *SAA* similar to the other APP is a biomarker of diseases (Eckersall and Bell, 2010). *M-SAA3* increases in cases of mastitis (Eckersall et al., 2006). In addition to inflammation and infection, *M-SAA3* increases in mid to late involution (Molenaar et al., 2009). Indeed, *M-SAA3* has antibacterial activity against *E. coli*, *Streptococcus uberis*, and *Pseudomonas aeruginosa*. Therefore, *M-SAA3* may have a role in protecting the mammary gland during mammary remodeling and infection and in neonatal calves' gastrointestinal tract as an antimicrobial against infection (Molenaar et al., 2009). *SAA3* is not only an APP but also is shown to be related to obesity and the respective insulin resistance (Lin et al., 2001); it also acts as chemoattractant in AT in which an increase in *SAA3* is linked to recruitment of monocyte-macrophages in AT (Han et al., 2007).

1.4. Conjugated linoleic acids

Conjugated linoleic acids are octadecadienoic acids (C18) containing two conjugated double bonds, separated by a single bond (Figure 7). Different CLA isomers exist; the main CLA isomers are the *cis*-9, *trans*-11 and the *trans*-10, *cis*-12 CLA isomer (Pariza et al., 2001).

1.4.1. Use of supplements containing conjugated linoleic acids

Nutritional quality is an important issue making food choice. In addition, consumers are increasingly aware of the links between diet, health, and disease prevention. Moreover, the importance of bioactive food components to enhance human health is increasingly discussed. CLA is one of the bioactive molecules present in animal derived foods. Ruminant products i.e. milk and meat contain CLA isomers. To increase the CLA concentration in these products, CLA is supplemented to the animal's diet. In addition, CLA reduces milk fat production in dairy cattle. Particularly, the *trans*-10, *cis*-12 CLA isomer is known for its milk fat reducing effect (Bauman et al., 2011).

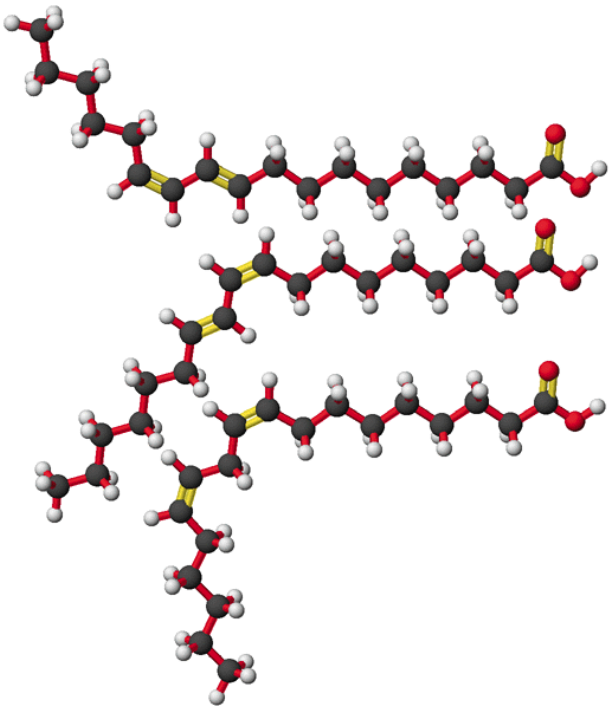

Figure 7. Isomers of conjugated linoleic acids (Adapted from James Cook lab web page http://www.cook.wisc.edu/).

Studies on CLA in dairy cows are divided to diet supplementation, abomasal infusion, and intravenous infusion of individual CLA isomers, mixtures of CLA isomers, or oils rich in linoleic acid (i.e. sunflower or safflower oils). CLA infusion in comparison to dietary supplementation of CLA causes a more severe milk fat depression (**MFD**). There is a curvilinear relationship between abomasal infusion of CLA and MFD. A maximum response of 50% reduction in milk fat yield via infusion of 7.5 g/d *trans*-10, *cis*-12 CLA isomer was achieved. One-half of the maximal effect was achieved by 3.5 g/d infusion of the *trans*-10, *cis*-12 CLA. Infusion of higher amounts of CLA into the abomasum caused a weak additional MFD response (de Veth et al., 2004). Lactating cows use the majority of energy intake for milk production. The energy required for milk fat synthesis is one-half of the energy required for milk production. Therefore, the CLA induced MFD effect could be used effectively to reduce the energy requirements for milk production (de Veth et al., 2004). A meta-analysis was conducted on a data set which was compiled from 14 experiments involving abomasal infusion of CLA isomers for more than 4 days and indeed having a MFD higher than 25%. The results showed that dry matter intake decreases by 1.5 kg/d during CLA induced MFD. The dry matter intake reduction accounts for a part of the energy spared from reduced milk fat synthesis (Harvatine et al.,

2009). In early lactation, the energy spared by MFD can be used for higher yield of milk and/or protein (Bauman et al, 2008). In dairy cows, MFD is considered as a specific effect of CLA at the mammary gland; both short and long term application of CLA to induce MFD had no effect on plasma concentrations of glucose, NEFA, BHB, insulin, IGF-1, growth hormone, and *LEP* in the majority of cases (Baumgard et al., 2002; Baumgard et al., 2000; Castaneda-Gutierrez et al., 2005; de Veth et al., 2006; Perfield II et al., 2002). In conclusion, homeostatic responses seem unaltered. In monogastric animals, CLA are also known for reducing body fat. Plourde et al. (2008) summarized the results of different studies about CLA induced body fat changes in humans and in mice and stressed the discrepancies related to substantially different experimental designs used in humans as compared to animal studies: the adiposity reducing effect of CLA is consistent in mice studies. In dairy cows receiving CLA supplements, body fat seems largely unaffected as evident from various indicators for body fat content and lipolysis at least during short term treatment (Baumgard et al., 2002) or treatment including a 4 week a.p. and a 5 week p.p. interval (Kay et al., 2006). However, when comparing fat cell size in different s.c. and visceral (v.c.) fat depots from CLA treated cows vs. a control group, we recently observed a decrease of adipocyte size with CLA treatment in 5 out of 6 different depots (Akter et al., 2011) whereas fat depot mass was decreased by CLA only in the retroperitoneal fat depot at 105 DIM (von Soosten et al., 2011; Akter et al., 2011). However, reduced mammary lipid synthesis during CLA treatment is consistent with the decreased lipid synthesis and body fat effects in other models, but the cellular responses and mechanisms are quite different, as reviewed by Bauman et al. (2008). The CLA doses used to elicit reductions in body fat are much greater than the doses used to reduce milk fat synthesis in the dairy cow (about 0.5 vs. 0.05% of the diet), and the dose used to induce MFD in the cow is expected to be much lower than the effective dose required to elicit direct AT responses in monogastrics.

1.4.2. CLA supplementation and alteration of gene expression

Genes related to CLA induced MFD in the mammary gland are well documented (Bernard et al., 2008). CLA (*trans*-10, *cis*-12) induced MFD was accompanied by a dramatic reduction (> 35%) of the mRNA abundance of enzymes involved in mammary uptake and intracellular trafficking of fatty acids [lipoprotein lipase (***LPL***) and fatty acid binding protein (***FABP***)], de novo FA synthesis [acetyl-CoA carboxylase (***ACC***) and fatty acid synthase (***FAS***)], desaturation [stearoyl-CoA desaturase (***SCD***)], and esterification

[glycerol-3 phosphate acyl transfrase (***GPAT***) and acyl glycerol phosphate acyl transfrase (***AGPAT***)]. Similarly, intravenous administration of *trans*-10, *cis*-12 CLA, either from 2 to up to 6 g/day (Viswanadha et al., 2003) or 10 g/day (Harvatine & Bauman, 2006), depressed milk fat yield. In the latter study (Harvatine & Bauman, 2006), a joint decrease observed in the expression of genes involved in mammary uptake of fatty acids (*LPL*), de novo fatty acid synthesis (***FASN***), and the regulation of lipid metabolism e.g. sterol regulatory element binding transcription factor 1 (***SREBP1***), thyroid hormone responsive spot 14 (***THRSP*** or ***S14***), insulin induced gene 1 (***INSIG1***).

In s.c. fat depot of dairy cows, effects of CLA on gene expression showed that expression of genes related to lipid synthesis i.e. enzymes like *LPL*, *SCD*, *FAS*, and *FABP4* increased 4 days after abomasal infusion of 7.5 g/d *trans*-10, *cis*-12 CLA. An important nuclear receptor and regulator of lipid synthesis, *PPARγ*, was also increased due to abomasal CLA infusion (Harvatine et al., 2009).

In monogastrics, CLA are known as ligands for *PPARs* (Bensinger and Tontonoz, 2008). Supplementation with the *trans*-10, *cis*-12 CLA isomer induced severe hepatic steatosis in mice with less effect in rats and hamsters. Regardless of species, lower body adiposity is observed when hepatic steatosis happens suggesting that the liver is not able to oxidize fatty acids mobilized from AT (Vyas et al., 2012). CLA (*trans*-10, *cis*-12) reduced the cellular and secreted *ADIPOQ* protein in the murine adipocyte 3T3-L1 cell line (Miller et al., 2008) and decreased *LEP* mRNA abundance in AT from rats (Gudbrandsen et al., 2009). Analogous findings for circulating *LEP* could not be established in dairy cattle (Baumgard et al., 2002; Block et al., 2003). Dietary supplementation of an equal mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers fed to pigs had no effect on *LEPRB* immunofluorescence intensity in AT (Di Giancamillo et al., 2009) although *LEP* expression measured using Western blotting and by immunofluorescence intensity was lowered. To our knowledge, there is no information available on CLA effects on *LEPRB* expression in cattle.

1.4.3. CLA supplementation and insulin sensitivity

Using an RQUICKI index which is calculated from blood metabolites (glucose and NEFA) and insulin concentrations, insulin sensitivity in cows can be estimated (Holtenius and Holtenius, 2007). The majority of studies about CLA supplementation to cows' diets reported that the blood concentrations of insulin (Baumgard et al., 2002, Baumgard et al., 2000, Castaneda-Gutierrez et al., 2005, de Veth et al., 2006), glucose, BHB, and NEFA

are not affected (Bauman et al., 2011) . Exceptionally, Baumgard et al. (2000) and Selberg et al. (2004) reported that NEFA and IGF-1 concentrations increased in serum of cows infused abomasally with the *trans*-10, *cis*-12 CLA isomer. Long term administration of a mixture of CLA isomers (2 weeks a.p. through 20 weeks p.p.) is only reported once (Bernal-Santos et al., 2003). Unfortunately, insulin was not measured in their study. In a mouse model, an increase in insulin release capacity of islets of Langerhans in CLA (*trans*-10, *cis*-12) fed mice was reported (Poirier et al., 2005) and was explained by an increase in ß-cell mass and number. Similarly, obese men treated with *trans*-10, *cis*-12 CLA showed hyperproinsulinemia (Risérus et al., 2004). Indeed, supplying a 50:50 mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers increased serum insulin concentrations in women (Raff et al., 2009).

1.4.4. CLA supplementation and APP

Conjugated linoleic acids and their relation to inflammatory cell signaling were recently reviewed by Reynolds and Roche (2010) addressing isomer specific effects of CLA on different *ILs*, *TNFs*, interferon gamma. Less is known about the contribution of CLA to APP in particular Hp and *SAA*. Obese rats treated with a mixture of CLA isomers had reduced Hp serum concentrations but Hp mRNA in liver was unchanged (Noto et al., 2007). It was suggested that the anti-inflammatory effect of CLA occurs at the level of AT although the technique used by Noto et al. (2007) for mRNA quantification was not sensitive enough to detect Hp mRNA in AT. A reducing CLA effect on *TNF-α* mRNA and protein in AT was also observed (Noto et al., 2007). In another study (Silvestre et al., 2011), higher concentrations of Hp and fibrinogen (one of the APP) were observed in plasma of cows fed with safflower oil (rich in linoleic acid) vs. palm oil (mainly containing saturated fatty acids). In growing heifers, Hp was decreased by addition of a rumen protected poly unsaturated fatty acid when compared with a non-supplemented diet (Araujo et al., 2010). Other inflammatory proteins e.g. alpha-1-acid glycoprotein were not affected by CLA supplementation in piglets, although serum lysozyme and total serum immunoglobulin G were higher (Corino et al., 2002). *SAA* and pro-inflammatory cytokines i.e. *TNF-α*, *COX-2*, and *IL-1ß* were down-regulated by *cis*-9, *trans*-11 CLA vs. butter fat in hamsters (Valeille et al., 2005).

In contrast to the studies in monogastrics, majority of the factors we already discussed are scarcely investigated in dairy cattle and in some cases no information is available. In early lactation, dairy cows experience different levels of NEB and reduced insulin sensitivity.

The cases of reduced insulin sensitivity addressed in monogastrics are in animal models of obesity, type 2 diabetes, or high fat diets which cannot be conveyed to dairy cattle. Therefore, understanding the pattern of the aforementioned factors related to insulin sensitivity and inflammatory system of dairy cattle and defining the contribution of dietary supplementation with CLA to the pathophysiological status of dairy cattle is highly relevant. To be able to address these aspects, we defined our objectives in next section.

2. Objectives

Herein, the expression of selected adipokines, one classical (*LEP* mRNA and protein) and one yet scarcely characterized in cattle (*ADIPOQ* mRNA) together with their respective receptors (*LEPRB*, *ADIPOR1*, and *ADIPOR2* mRNA) was recorded. The mRNA abundance of nuclear receptors *PPARγ* and *PPARγ2* as regulators of gene expression, of the proinflammatory cytokines *IL-6* and *TNF-α*, as well as of the APP Hp and *SAA3* was quantified. The serum concentrations of Hp, insulin, NEFA, and glucose were determined and an index estimating insulin sensitivity (RQUICKI) was calculated from the latter 3 variables. In two trials, we studied cows in 1st lactation from day 1 to 105 p.p. or cows in higher lactation number from 21 days a.p. until day 252 p.p. The aforementioned cows received a commercially available 1:1 mixture of *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers during a period of 105 (primiparous cows) or 182 (pluriparous cows) days in milk, respectively. We hypothesized that based on the extent of NEB, cows in 1st vs. higher lactation number might divergently respond to CLA with regard to the target genes. CLA might affect insulin sensitivity in different tissues of dairy cows. Indeed, the serum factors related to insulin sensitivity in dairy cows might be affected similar to monogastrics. With regard to the aforementioned factors, the objectives of this study were:

1) to characterize physiological changes of either mRNA expression or protein concentration of the aforementioned factors in a whole lactation cycle of dairy cows,
2) to investigate effects of a dietary supplementation of CLA during the first 105 or 182 days in milk,
3) to record potential post-treatment effects of CLA supplementation,
4) to compare the mRNA expression of the target genes between 3 different v.c. and 3 s.c. adipose depots, and in liver, skeletal muscle, and mammary gland, and
5) to discover possible differences between cows in 1st vs. higher parities.

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Technical note: **Identification of reference genes for gene expression studies in different bovine tissues focusing on different fat depots**

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ABSTRACT

Selection of stable reference genes (REF) is important in real-time PCR data normalization. Bovine tissues such as the mammary gland, liver, muscle, and s.c. fat from the tail head have been thoroughly explored for stable REF, whereas fewer reports exist for other fat depots. Therefore, a suitable combination of REF was tested for different tissues of dairy cattle. Holstein dairy heifers (n = 25) were supplemented (100 g/d) with a control fat (n = 15) without conjugated linoleic acids or with rumen-protected conjugated linoleic acids (n = 10) from the day of calving until slaughter at 1, 42, or 105 d postpartum (n = 5, 10, and 10, respectively). Samples from 6 fat depots (omental, mesenterial, retroperitoneal, s.c. tail head, s.c. withers, and s.c. sternum), liver, semitendinosus muscle, and mammary gland were collected. The REF mRNA were quantified and their stability was analyzed using geNorm^{plus}. The 3 most stable REF in individual fat tissues and muscle were *EMD* (emerin), *POLR2A* (RNA polymerase II), and *LRP10* (lipoprotein receptor-related protein 10); in mammary gland were *MARVELD1* (marvel domain containing 1), *EMD*, and *LRP10*; and in liver were *HPCAL1* (hippocalcin-like 1), *LRP10*, and *EIF3K* (Eukaryotic translation initiation factor 3). The 3 most stable REF in s.c. fat were *EMD*, *LRP10*, and *EIF3K*; in visceral fat were *POLR2A*,

LRP10, and *MARVELD1*; and for all 6 adipose tissues were *LRP10*, *EIF3K*, and *MARVELD1*. When the mammary gland was added to the 6 adipose depots, at least 5 REF (*LRP10*, *POLR2A*, *EIF3K*, *MARVELD1*, and *HPCAL1*) were needed to reach the threshold of 0.15. Addition of liver to the above-mentioned tissues increased the *V* value. The data improve the comparison of gene expression between different fat depots. In each case, *GAPDH* had the lowest stability value.

Key words: reference gene, adipose tissue, normalization, cattle

Technical Note

Real-time PCR is the most accurate and fast method to analyze changes in gene expression, even for extremely low abundance genes (Bustin and Mueller, 2005). The steps from tissue sample preparation to running the real-time PCR comprise different kinds of technical variation (Bustin, 2010). It is possible to correct for such experimental inaccuracies by using at least 3 of the most stable internal control genes (reference genes, **REF**; Vandesompele et al., 2002), which are expected to be stably expressed in different tissues. Treatment and physiological conditions should have no effect on REF stability (Huggett et al., 2005). In addition, REF should not be co-regulated (Piantoni et al., 2008); otherwise, a bias in pairwise comparisons between REF will occur. Completely stable REF do not exist (Vandesompele et al., 2002) and co-regulation between REF cannot be entirely excluded. Recently, it was recommended that REF be chosen from microarray databases (Hruz et al., 2011) or studies involving wide screen gene expression profiling (Kadegowda et al., 2009) due to the broader view of genes stably expressed in different tissues. Subsequent verification by real-time PCR has a higher chance of achieving valid results.

Scientists have attempted to define some REF in subcutaneous (**s.c.**) tail head and mesenterial adipose tissue (**AT**; Mukesh et al., 2010), liver (Janovick-Guretzky et al., 2007), and the mammary gland (Kadegowda et al., 2009) in cattle. However, well-defined, appropriate REF for comparing different bovine tissues, especially AT from different locations, are lacking. The objective was to introduce a set of REF for studies on different tissues of dairy cows evaluating potential effects of supplementation with conjugated linoleic acid (**CLA**). The basic animal experiment was described previously

(von Soosten et al., 2011). In brief, 5 of 25 Holstein dairy heifers were slaughtered at 1 DIM and the remainder (n = 20) were allocated randomly to either a control fat supplement (non-CLA FA mixture: Silafat, BASF, Ludwigshafen, Germany) or CLA supplementation (Lutrell Pure, BASF) at 100 g/d starting from 1 DIM until slaughter. Five heifers per group were slaughtered at 42 and 105 DIM. Samples from 3 visceral (**v.c.**) AT (omental, mesenterial, and retroperitoneal), 3 s.c. AT (tail head, withers, and sternum), liver, muscle (semitendinosus), pancreas, and mammary gland were immediately dissected after slaughter. The samples were snap-frozen in liquid nitrogen and stored at −80°C. Complete details about RNA extraction and purification and respective quality controls, cDNA synthesis, and relative quantification using real time PCR can be found in the supplemental materials (available at http://www.journalofdairyscience.org/).

Three REF (*EMD*, emerin; *MARVELD1*, marvel domain containing 1; and *EIF3K*, eukaryotic translation initiation factor 3, subunit K; Kadegowda et al., 2009) were selected on the basis of a previous bovine microarray study. The other REF (*LRP10*, lipoprotein receptor-related protein 10; *POLR2A*, RNA polymerase II; and *HPCAL1*, hippocalcin-like 1; Hosseini et al., 2010) were selected based on previous experiments in our laboratories. Leptin, as an exemplary gene of interest, and *GAPDH*, as one of the commonly used REF, were also analyzed, as described in Table 1.

The quantification cycle values were imported to qBASE^{plus} version 2.0 (Biogazelle, Ghent, Belgium) and all subsequent calculations and data quality controls were done based on this software (Hellemans et al., 2007). Average expression stability (***M***) and pairwise variation (***V***) values for determination of stable REF and number of REF were calculated using geNorm^{plus} (Biogazelle). Reference genes should have *M* and *V* values below the threshold levels of 1.5 and 0.15, respectively (Vandesompele et al., 2002). Ingenuity Pathways Analysis (Ingenuity, Redwood City, CA) was used to examine potential relationships between the chosen REF in the present study.

Non-normalized data (relative quantities based on amplicon standard curves) were analyzed by using the general linear model in SPSS 17.0 (SPSS Inc., Chicago, IL) to assess fixed effects of treatment, DIM, and the respective interaction on the relative mRNA abundance of each REF. Means were compared using the Tukey test with Bonferroni adjustment for multiple comparisons ($P < 0.05$). To compare total RNA concentrations harvested from different tissues at different DIM, the model was limited to the control group (a trend was defined at $0.1 > P \geq 0.05$). Leptin mRNA abundance was

analyzed using the same general linear model or nonparametric test based on results of homogeneity of variances. Pearson correlation was used to assess the relation between REF ($P < 0.05$).

The RNA integrity number was around 7.27 ± 0.12 for all fat tissues, liver, muscle, and the mammary gland, whereas the quality of RNA extracted from pancreas was poor (RNA integrity number = 2). Therefore, the data for pancreas are not shown. Table 1 shows the coefficients of variation (8.19 ± 0.36%) of the real-time PCR replicates per gene. The efficiency of the real-time PCR reactions was close to 100%, with R^2 values close to 1 (Table 1).

The *M* values of all analyzed REF in each individual tissue were below the defined threshold of 1.5 (Table 2); 20% of the *M* values exceeded 0.5, but none were higher than 0.79. Combining data from all s.c. fat samples and recalculating REF gene expression stability resulted in similar *M* values as reported for individual tissues. Combining v.c. AT or all fat depots led to increased *M* values up to 0.84. Adding mammary gland and liver to the fat tissue data resulted in an upper range of *M* values of 0.94 and 1.09, respectively. The highest *M* value was consistently observed for *GAPDH*.

To determine the optimal number of REF necessary for normalization of the gene of interest for individual tissues or a combination of tissues, *V* values were ascertained (Table 3). The 3 most stable REF in individual fat tissues were *EMD*, *POLR2A*, and *LRP10*; the addition of the fourth and fifth REF reduced *V* values by up to 0.03. For muscle tissue, a set of the 3 most stable REF comparable to that for individual fat tissues could be used, but the *V* value was 0.03 to 0.04 units higher. The 3 most stable REF for mammary gland were *MARVELD1*, *EMD*, and *LRP10*, and those for liver were *HPCAL1*, *LRP10*, and *EIF3K*. Addition of the next REF in each case reduced the corresponding *V* value (by 0.03 and 0.12, respectively). Combining different tissues increased the *V* values. In case of AT (6 fat depots, 3 s.c. AT or 3 v.c. AT), 3 REF were sufficient to achieve a *V* value <0.15 (as shown in Table 3), although the *V* value was higher compared with those of the individual fat depots. In these cases, addition of the next REF improved the *V* value, especially in case of v.c. AT (by 0.05 units). Addition of mammary gland data to the 6 different AT required the use of at least 5 REF to reach the threshold *V* value of 0.15. Liver included in the panel of tissues resulted in a *V* value of 0.155 by incorporation of the first 4 stable REF, and addition of fifth and sixth REF resulted in no improvement.

The advantage of using the most appropriate REF based on a lower *V* value is shown in

Figure 1A. The highest technical SE occurred when *GAPDH* was used for normalization. Using *EIF3K* as the most stable REF improved the technical SE. In each case, the lowest *V* value using 6 REF resulted in the lowest technical SE and addition of a seventh REF (*GAPDH*) increased the *V* value and led to additional technical SE. As shown in Figure 1B and 1C, the example for leptin as a gene of interest, using inappropriate REF biased the relative abundance of leptin in different treatments or tissues. For each sampling date, a numerically higher or lower (omental or s.c. sternum, respectively) abundance of leptin mRNA was obtained when *GAPDH* was used for normalization compared with other individual REF or sets of REF used for normalization. This difference was significant at 42 DIM within the CLA group when comparing *GAPDH* with *EIF3K* as the most stable REF. The SEM for leptin mRNA abundance by ANOVA was lower if the geometric mean of the 6 most stable genes was used instead of *GAPDH* in all fat depots, but not in mesenterial AT.

Statistical analysis of non-normalized relative quantities demonstrated that CLA had no effect on REF relative quantities in any of the tissues examined. Individual relative quantities of REF were selectively regulated by DIM. This regulation was limited to statistical differences between d 1 and d 42 or d 105 and in only some of the tissues. In mesenterial, omental, retroperitoneal, and s.c. sternum AT, no effect of DIM was observed. In s.c. tail head, *EIF3K*, *HPCAL1*, and *POLR2A* showed different values within DIM. In s.c. withers, all REF except *MARVELD1* and *GAPDH* were regulated by DIM. All REF were stable at d 1, 42, and 105 in liver tissue, except *MARVELD1*, which showed a significant difference between d 1 and d 42 or d 105. In the mammary gland and muscle tissue, all REF were regulated by DIM, except *MARVELD1* in the mammary gland and *GAPDH* and *HPCAL1* in muscle tissue.

Pathway analysis showed that *HPCAL1* directly regulates *GAPDH* via protein–protein interaction and *POLR2A* regulates *GAPDH* via protein–DNA interaction. Furthermore, *GAPDH* showed self-activation and protein–protein interaction. The remaining REF exhibited no direct co-regulation.

Purified RNA (ng/mg of tissue) was plotted against DIM (Figure 2). Total RNA yield was higher ($P < 0.05$) in samples from mesenterial AT, s.c. sternum AT, and from muscle tissue obtained on 105 DIM compared with that obtained on d 1 and 42. For retroperitoneal AT and mammary gland tissue, this difference was seen as a trend ($0.1 > P \geq 0.05$).

In general, the statistical analysis of raw data to detect a time effect was not suited for

selection of appropriate REF for longitudinal-type experiments (Bionaz and Loor, 2007). Therefore, the significant differences between d 1, 42, and 105 could be due to the reported RNA dilution effect described by Bionaz and Loor (2007), depending on differences in gene expression of other genes directly after parturition. In spite of the fact that the RNA extraction protocol we used was not optimized for this kind of comparison, effects of time of lactation on RNA yield were detected for mesenterial AT, s.c. sternum AT, and skeletal muscle. As demonstrated herein, the lack of CLA effect on all REF indicates that a simple statistical test is insufficient to evaluate REF stability. Taken together, these results support the need for pairwise comparisons of REF to assess the appropriate REF.

Only a few bovine AT mRNA expression studies (Hosseini et al., 2010; Mukesh et al., 2010) have described the use of more than 1 REF. Mukesh et al. (2010) introduced a panel of REF for s.c. tail head and mesenterial AT extracted from a microarray experiment on s.c. AT, in which the highest *M* value was 0.47. For s.c. and retroperitoneal AT, Hosseini et al. (2010) introduced some additional REF to the commonly used REF. The highest *M* value was around 0.5. Therefore, the list of stable REF recommended by our study is suitable for normalization of AT mRNA expression because the *M* value was around 0.31. Decreasing SEM for leptin mRNA data that were normalized based on the geometric mean of 6 most stable REF compared with *GAPDH* supports the finding that this set of 6 REF yields a more accurate normalization of the gene of interest. In addition, we introduced a panel of REF for s.c. withers, s.c. sternum, and omental AT, which have not been intensively explored for stable REF. Both experiments (Hosseini et al., 2010; Mukesh et al., 2010) demonstrated that the resulting pairwise variation (*V* value) was 0.20 when comparing the 2 fat depots (s.c. and retroperitoneal AT or s.c. and mesenterial AT, respectively), which is relatively high. By using the panel of REF illustrated here for 3 s.c., 3 v.c., or all 6 AT together, the pairwise variation would be 0.14 or 0.11, using the 3 or 4 most stable REF. This gives an opportunity to isolate physiologically important differences that might be lost because of technical SE.

The REF suggested herein for bovine liver mRNA expression, although not derived from microarray studies, were stable enough to be recommended for normalization. Therefore, in addition to Janovick-Guretzky et al. (2007), who selected ribosomal protein S9 out of 4 commonly and 4 less used REF, our results extend the pool of stable REF for liver. For fat tissues, it is possible to use the stable REF identified for mammary gland by Kadegowda et al. (2009), but they cannot be used for liver.

The REF *EMD*, *MARVELD1*, and *EIF3K*, which were recommended by Kadegowda et al. (2009), were reconfirmed in the current study as stable REF for the bovine mammary gland. In addition, *LRP10*, *POLR2A*, and *HPCAL1* had *M* values close to those of *EMD* and *MARVELD1* but were lower than that for *EIF3K*, and adding LRP10, POL2A, and HPCAL1 to the panel reduced the pairwise variation. In addition to the useful stable REF provided by Perez et al. (2008) and Graugnard et al. (2009), the proposed REF were stable in muscle tissue with respect to *M* and *V* values.

In normalization processes, how many REF to use is an important consideration. Increasing the number of stable REF will result in lower *V* and *M* values and will thus lower the technical SE. Reducing the technical SE will result in lower standard error of mean. Herein, we demonstrate a new set of REF for gene expression profiling in bovine tissues. The key difference compared with previous studies is the greater stability of REF in the current study, which permits isolation of small but physiologically important differences. This study allows comparison between different tissues by providing adequate stable REF. Moreover, REF were suggested for some of the adipose depots for which stable REF have not yet been investigated. We suggest abandoning the use of *GAPDH* as the sole REF if its stability is not approved by one of the known algorithms such as geNorm.

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Table 1. Characteristics of the primers and the real-time PCR conditions

Primers[1]	Sequence (5'-3')	NIH GenBank accession no.	bp[2]	Amount (nmol)	Mean Cq value[3]	Annealing[4]	R^2	Efficiency	Mean CV (%)
EIF3K									
Forward	CCAGGCCCACCAAGAAGAA	NM_001034489	125	400	22.87	45 s at 59˚C	0.99	97.54	9.52
Reverse	TTATACCTTCCAGGAGGTCCATGT								
LRP10									
Forward	CCAGAGGATGAGGACGATGT	Bc149232	139	400	21.90	30 s at 61˚C	0.99	101.1	7.95
Reverse	ATAGGGTTGCTGTCCCTGTG								
POLR2A									
Forward	GAAGGGGGAGAGACAAACTG	X63564	86	800	23.86	60 s at 60˚C	0.98	97.4	7.97
Reverse	GGGAGGAAGAAGAAAAAGGG								
GAPDH									
Forward	AATGGAAAGGCCATCACCATC	U85042	204	400	19.02	75 s at 59˚C	0.99	98.2	6.57
Reverse	GTGGTTCACGCCCATCACA								
EMD									
Forward	GCCCTCAGCTTCACTCTCAGA	NM_203361	100	400	22.07	45 s at 59˚C	0.99	101.7	9.11
Reverse	GAGGCGTTCCCGATCCTT								
MARVELD1									
Forward	GGCCAGCTGTAAGATCATCACA	NM_001101262	100	400	22.61	45 s at 59˚C	0.99	101.2	8.28
Reverse	TCTGATCACAGACAGAGCACCAT								
HPCAL1									
Forward	CCATCGACTTCAGGGAGTTC	NM_001098964	99	400	23.98	30 s at 60˚C	0.99	97.9	7.96
Reverse	CGTCGAGGTCATACATGCTG								
Leptin									
Forward	GACATCTCACACACGCAG	U62123	183	400	29.23	30 s at 60˚C	0.99	88.6	7.45
Reverse	GAGGTTCTCCAGGTCATT								

[1]*EIF3K*: Eukaryotic translation initiation factor 3, subunit K; *LRP10*: Lipoprotein receptor-related protein 10; *POLR2A*: RNA polymerase II; *GAPDH*: Glyceraldehyde-phosphate-dehydrogenase; *EMD*: Emerin; *MARVELD1*: Marvel domain containing 1; *HPCAL1*: Hippocalcin-like 1.
[8]bp: base pairs.
[2]Amplicon size.
[3]Cq = quantification cycle; results based on slaughter experiment.
[4]Initial denaturation = 10 min at 95˚C; denaturation = 30 s at 95˚C; extention = 30 s at 72˚C, except for *POLR2A* (60 s at 72˚C).

Table 2. Ranking of the most stable reference genes (*M* values) that can be used for normalization in different bovine tissues[1]

Tissue	Reference gene[2]													
	EIF3K		*LRP10*		*POLR2A*		*GAPDH*		*EMD*		*MARVELD1*		*HPCAL1*	
	M	Rank	*M*	Rank	*M*	Rank	*M*	Rank	*M*	Rank	*M*	Rank	*M*	Rank
Fat tissue														
Omental	0.33	4	0.30	3	0.29	2	0.54	-	0.26	1	0.36	5	0.42	-
Mesenterial	0.37	6	0.26	1	0.30	4	0.53	-	0.26	2	0.27	3	0.34	5
Retroperitoneal	0.35	5	0.30	3	0.30	2	0.56	-	0.28	1	0.33	4	0.41	-
Subcutaneous tail head	0.35	5	0.26	2	0.27	3	0.53	-	0.25	1	0.31	4	0.38	6
Subcutaneous withers	0.37	4	0.27	1	0.30	3	0.73	-	0.27	2	0.45	5	0.52	6
Subcutaneous sternum	0.27	3	0.27	2	0.30	4	0.47	-	0.26	1	0.37	6	0.34	5
Non fat tissues														
Muscle	0.45	4	0.35	1	0.38	3	0.78	-	0.36	2	0.64	-	0.55	-
Mammary gland	0.31	4	0.28	3	0.36	5	0.58	-	0.26	2	0.26	1	0.34	6
Liver	0.38	3	0.32	2	0.33	4	0.43	5	0.50	-	0.56	-	0.28	1
Combination of tissues														
Subcutaneous fat	0.34	3	0.32	2	0.56	6	0.74	-	0.30	1	0.42	4	0.49	5
Visceral fat	0.48	4	0.44	2	0.42	1	0.69	-	0.82	-	0.46	3	0.52	5
Fat	0.51	2	0.51	1	0.56	4	0.84	-	0.72	6	0.51	3	0.63	5
Fat + Mammary gland	0.57	3	0.55	1	0.57	2	0.94	-	0.79	6	0.65	4	0.72	5
Fat + Mammary gland + liver	0.60	2	0.56	1	0.61	3	1.09	-	0.79	-	0.94	-	0.66	4

[1]n = 25

[2]See Table 1 for description of genes.

[3]Dash indicates rank of the reference genes with the lowest stability that should not be used for normalization according to pairwise variation in Table 3.

Table 3. Proper combination of the most stable reference genes based on the pairwise comparisons (*V* values)[1]

Tissue	Order of reference genes stability[2]	V2/3	V3/4	V4/5	V5/6	V6/7
Fat tissues						
Omental	*EMD, POLR2A, LRP10, EIF3K, MARVELD1*	0.101	0.081	0.072	-[3]	-
Mesenterial	*LRP10, EMD, MARVELD1, POLR2A, HPCAL1, EIF3K*	0.088	0.072	0.071	0.061	-
Retroperitoneal	*EMD, POLR2A, LRP10, MARVELD1, EIF3K*	0.096	0.076	0.068	-	-
Subcutaneous tail head	*EMD, LRP10, POLR2A, MARVELD1, EIF3K, HPCAL1*	0.091	0.081	0.069	0.067	-
Subcutaneous withers	*LRP10, EMD, POLR2A, EIF3K, MARVELD1, HPCAL1*	0.109	0.102	0.108	0.098	-
Subcutaneous sternum	*EMD, LRP10, EIF3K, POLR2A, HPCAL1, MARVELD1*	0.086	0.070	0.071	0.063	-
Non fat tissues						
Muscle	*LRP10, EMD, POLR2A, EIF3K*	0.136	0.119	-	-	-
Mammary gland	*MARVELD1, EMD, LRP10, EIF3K, POLR2A, HPCAL1*	0.099	0.074	0.069	0.056	-
Liver	*HPCAL1, LRP10, EIF3K, POLR2A, GAPDH*	0.119	0.097	0.087	-	-
Combination of tissues						
Subcutaneous fat	*EMD, LRP10, EIF3K, MARVELD1, HPCAL1, POLR2A*	0.120	0.113	0.112	0.105	-
Visceral fat	*POLR2A, LRP10, MARVELD1, EIF3K, HPCAL1*	0.152	0.105	0.103	-	-
Fat	*LRP10, EIF3K, MARVELD1, POLR2A, HPCAL1, EMD*	0.149	0.133	0.133	0.132	-
Fat + Mammary gland	*LRP10, POLR2A, EIF3K, MARVELD1, HPCAL1, EMD*	0.175	0.167	0.145	0.136	-
Fat + Mammary gland +liver	*LRP10, EIF3K, POLR2A, HPCAL1*	0.194	0.155	-	-	-

[1]n = 25

[2]Reference gene stability decreases from left to right. reference gene stability decreases. See Table 1 for description of genes.

[3]Dash indicates whether inclusion of next gene increased pairwise variation values.

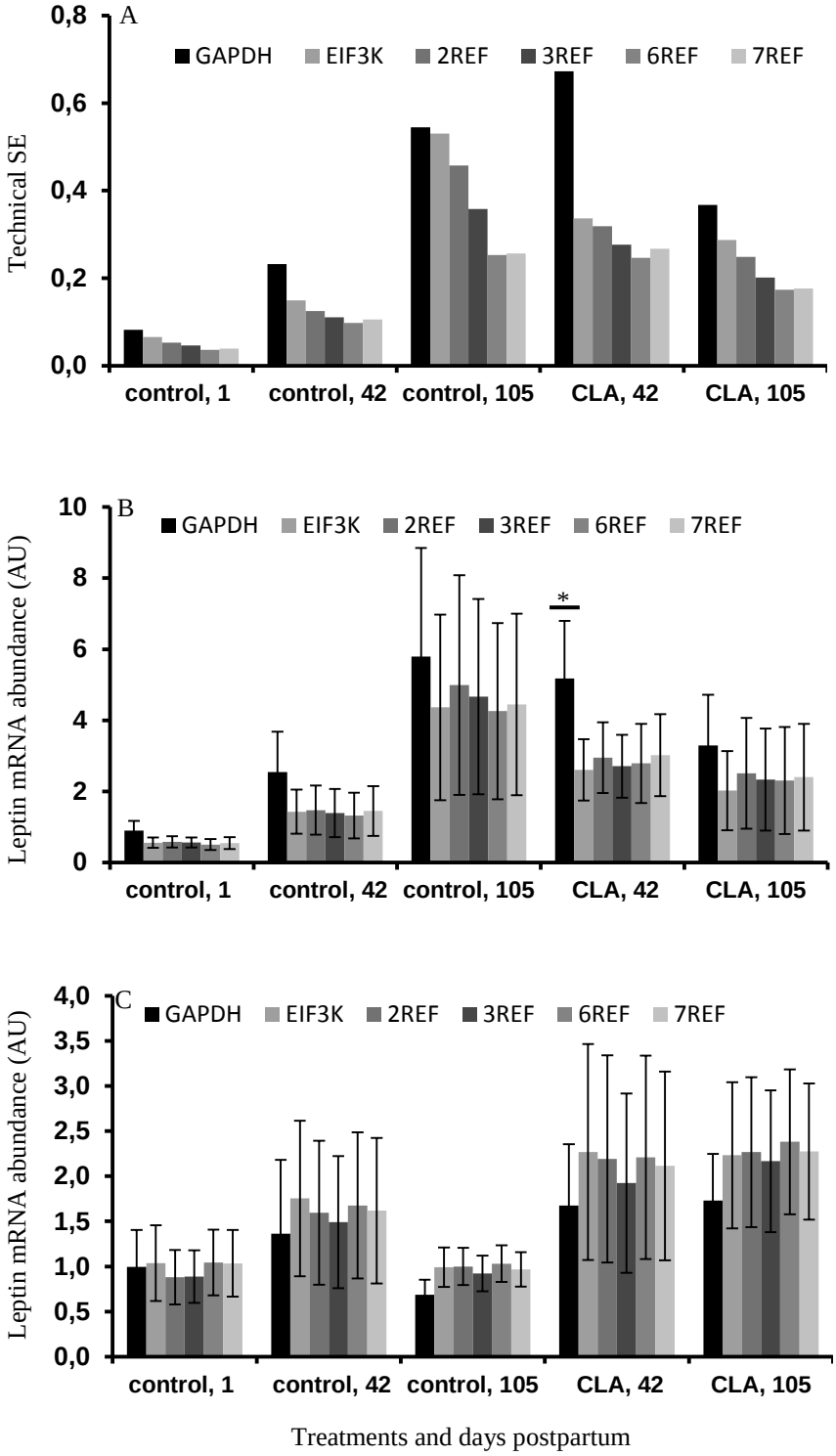

Figure 1. Technical SE in omental adipose tissue (A), and leptin mRNA abundance in omental (B) and s.c. sternum (C) adipose tissues of heifers supplemented with 100 g/d conjugated linoleic acids (CLA) or a control fat supplement (Control) on d 1, 42 and 105 postpartum. Data were normalized based on *GAPDH* or *EIF3K* or the geometric mean of 2 reference genes (REF; *EIF3K* and *LRP10*), 3 REF (*EIF3K*, *LRP10*, and *MARVELD1*), 6 REF (*EIF3K*, *LRP10*, *MARVELD1*, *POLR2A*, *HPCAL1*, and *EMD*), or 7 REF (*EIF3K*, *LRP10*, *MARVELD1*, *POLR2A*, *HPCAL1*, *EMD*, and *GAPDH*). Asterisk indicates differences ($P < 0.05$) for leptin mRNA abundance (mean ± SE) between different REF and groups of REF used for normalization. AU: arbitrary units.

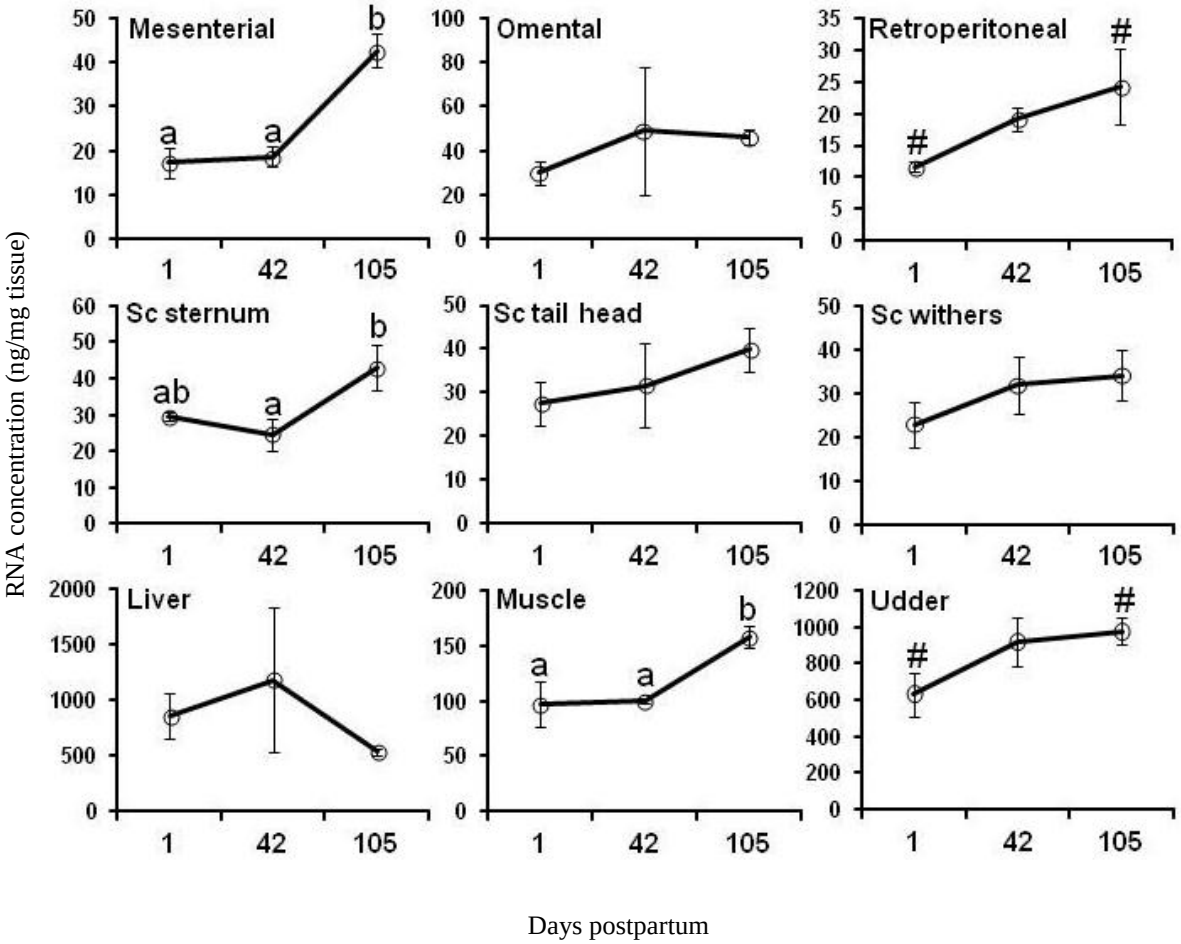

Figure 2. Total RNA harvested from different tissues of heifers fed with a control fat supplement from d 1 until d 105 postpartum. Different letters designate differences ($P < 0.05$); trends ($0.1 > P > 0.05$) are indicated by #.

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Bovine haptoglobin as an adipokine: Serum concentrations and tissue expression in dairy cows receiving a conjugated linoleic acids supplement throughout lactation

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ABSTRACT

The present study aimed to characterize serum haptoglobin (Hp) concentrations throughout an entire lactation period in both primi- and multiparous cows and to compare them to the Hp mRNA expression in liver and - in view of Hp being potentially an adipokine - also in different subcutaneous (s.c.) and visceral fat depots. In addition, potential anti-inflammatory effects of long-term supplementation with conjugated linoleic acids (CLA) were evaluated by assessing Hp. Trial 1 comprised 33 cows and 16 Holstein heifers from day 21 ante partum until day 252 postpartum. The animals received 100 or 50 g/day CLA or a control fat supplement. Blood samples and biopsy (tail head fat and liver) samples were collected. Trial 2 included 25 Holstein heifers, 5 animals were

slaughtered on the day of parturition, the remaining animals were allocated to either CLA (100 g/day, n = 10) or control fat supplement (n = 10) and slaughtered on days 42 and 105 postpartum, respectively. At slaughter, fat samples were collected from 3 different visceral depots, 3 s.c. depots and from liver tissue. Results indicated no effects of CLA on serum Hp and liver Hp mRNA for both trials and on Hp mRNA in biopsies from s.c. tail head fat. In omental and s.c. withers fat from trial 2, CLA reduced Hp mRNA on both day 42 and day 105. Hp mRNA was detectable in fat tissues from both trials with abundance values being significantly lower than in liver. The Hp mRNA abundance in the s.c. fat depots was generally higher than in the visceral depots. Haptoglobin mRNA abundance in the different tissues from trial 2 was correlated whereby all s.c. depots were interrelated. The evidence of Hp mRNA expression in adipose tissues and the presence of Hp-immune staining in histological fat sections confirm that Hp can be classified as a bovine adipokine. The lack of an evident relationship between circulating Hp concentrations and normal body fat portions in dairy cattle demonstrates that varying degrees of adiposity are not confounding factors when using Hp as inflammatory marker. The physiological changes in serum Hp concentration seem to be limited to parity and parturition. In view of the lack of effects of CLA on serum Hp concentrations, the observed reaction in two out of six different fat depots seems of marginal importance for the organisms as an entity.

Key words: Adipose tissue, Conjugated linoleic acids, Dairy cow, Haptoglobin.

1. Introduction

Haptoglobin (Hp) is a major acute phase protein (APP) in cattle with serum concentrations < 20 μg/mL in healthy animals and 100 to 1000-fold increases in response to immune stimulation (Skinner et al., 1991; Godson et al., 1996). In dairy cows, Hp has been used as a marker for various diseases including typical production diseases such as mastitis and fatty liver syndrome (Eckersall and Bell, 2010). Haptoglobin is produced mainly by the liver, but extrahepatic expression such as mammary gland, leukocytes, forestomach and abomasum has also been demonstrated in cattle (Thielen et al., 2005; Thielen et al., 2007; Dilda et al., 2011). In humans, serum Hp concentrations are increased in obese and diabetic patients (Engstroem et al., 2003), correlate with body fat content in obese states (Chiellini et al., 2004; Doumatey et al., 2009), and white adipose tissue has indeed been identified as a site of Hp expression in several monogastric species, e.g. in mice, dogs and pigs (Friedrichs et al., 1995; Eisele et al., 2005; Ramsay et

al., 2010) and in man (Fain et al., 2004). Haptoglobin can thus also be classified as an adipokine in these species. The term adipokine comprises proteins synthesized and secreted from adipose tissue (AT) such as classical cytokines, growth factors, hormones, and also APP. Adipokines exert pleiotropic functions, mainly related to energy metabolism and the immune system. The increased production of inflammatory cytokines and APP by AT in obesity is commonly related to localized events within the expanding fat depots (Trayhurn and Wood, 2004) whereby differences between visceral (v.c.) and s.c. depots exist (Fain et al., 2010). Although obesity rarely occurs in dairy cows it is known that overconditioned cows are more likely to develop reproductive problems (Gearhart et al., 1990) and that massive mobilization of white AT fat mass during the transition from pregnancy to peak lactation is linked to a period of insulin resistance, reduced feed intake, negative energy balance, hypocalcaemia and reduced immune function (LeBlanc, 2010). A direct involvement of AT into immune function in cows is likely since expression of proinflammatory cytokines and of another APP, serum amyloid A3, has recently been demonstrated for both s.c. and mesenterial fat depots together with their *in vitro* responsiveness towards a proinflammatory stimulus (Mukesh et al., 2010). In view of the established diagnostic relevance of serum Hp concentrations, we aimed to elucidate their relation with body condition and with the potential expression of this APP in different adipose depots, thus pointing out the importance for Hp being a bovine adipokine. We therefore characterized the Hp concentrations throughout an entire lactation period in both primiparous and multiparous cows and compared them to the Hp mRNA expression in liver as well as in six different s.c. and v.c. fat depots. Using immunohistochemistry, we extended the Hp analyses in AT to the protein level. In addition to the physiological changes during lactation, we also tested the effects of dietary supplementation with conjugated linoleic acids (CLA) on Hp in serum, liver and fat. Supplements containing rumen-protected formulations of CLA mainly contain the *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA isomers (Kramer et al., 2004), and reduce milk fat synthesis whereas the effects on body fat seem to be mostly limited to monogastric species since indicators related to lipolysis and energy homeostasis (non esterified fatty acids (NEFA), leptin, insulin-like growth factor 1, adrenergic responsiveness) reportedly remain mostly unchanged in CLA supplemented dairy cows (Bauman et al., 2008). However, recent reports indicate that adipocyte size in dairy cows is decreased in response to CLA (Akter et al., 2011) whereas fat mass was only affected in the retroperitoneal depot (von Soosten et al., 2011). Based on results from monogastric

species, CLA might also exert immunomodulatory actions by attenuating the proinflammatory state in adipose tissue that predisposes to insulin resistance and by suppressing inflammatory responses as demonstrated in a variety of animal models in an isomer specific manner (Hontecillas et al., 2002; Jaudszus et al., 2008; Moloney et al., 2007). In context with these immunomodulatory actions of CLA, decreased blood concentrations of Hp were demonstrated in obese, insulin-resistant rats (Noto et al., 2007). In our study we aimed (1) to assess whether Hp can be designated as an adipokine in cattle, (2) to compare Hp expression in different fat depots, (3) to test for relations between Hp serum concentrations and Hp expression in liver and adipose tissues and (4) to evaluate potential effects of CLA supplements on circulating Hp concentration and Hp tissue expression.

2. Materials and methods

2.1. Animals, trials and treatments

The regulations of the German Animal Welfare Act (TierSchG) in its respective current edition were met. All animal experiments were approved by the lower saxony state office for consumer protection and food safety (LAVES, file no. 33.11.42502-04-071/07, Oldenburg, Germany). All animals were housed in free stall barns and milked twice daily in a milking parlor at the experimental station of the Friedrich Loeffler Institute, Federal Research Institute for Animal Health, Braunschweig, Germany. Two trials were performed in this study, and all the diets were formulated according to the recommendation of the German Society of Nutrition Physiology (GfE, 2001) and are described in detail by Pappritz et al. (2011, trial 1) and von Soosten et al. (2011, trial 2). If signs of disease were noted during milking by the milking staff or during the daily animal control by the stockmen and the veterinarian, respectively, they were filed in a list. When indicated, the cows were attended by the veterinarian. For the present evaluation, diagnostic findings were only classified as positive (diseased) or negative (non-diseased).

2.1.1. Trial 1

German Holstein-Friesian cows (n=33) and heifers (n=16) were studied from day 21 ante partum until day 252 of the subsequent lactation. The animals were fed *ad libitum* over the entire experimental period with a partial mixed ration (PMR) consisting of 63% silage and 37% concentrate (6.8 MJ NE_L/kg DM) on dry matter (DM) basis. On day 1

after calving, the animals were randomly allocated to three groups balanced for lactation number (average 1.9 ± 0.1). For the cows, the milk yield of the previous lactation (5797 ± 122 kg, 200 days in milk) and live weight (627 ± 9 kg) were also considered. The animals received 4 kg of additional concentrate (8.8 MJ NE_L/kg DM) containing either 100 g/day of a lipid encapsulated CLA supplement (Lutrell® Pure, BASF SE, Ludwigshafen, Germany; CLA-100: 11 cows and 5 heifers) or 50 g CLA supplement and 50 g of a control fat supplement (Silafat®, BASF SE; CLA-50: 11 cows and 6 heifers). The control group received 100 g of control fat supplement (CTR: 11 cows and 5 heifers). The animals of the CLA-50 group consumed 4.3 and 4.0 g/day of the *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA isomers, respectively (calculated, based on the analyzed proportions in the concentrate). In the CLA-100 group, the animals consumed 7.6 g/day each of the *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA isomers. In the CTR fat supplement these isomers were substituted by stearic acid. Supplements were fed from day 1 until day 182 postpartum.

Blood samples were obtained from each animal via jugular venipuncture on days -21, -14, -7, 1, 7, 14, 21, 35, 49, 70, 105, 140, 182, 189, 196, 210, 224, 238 and 252 relative to parturition. After centrifugation (10 min, 3000 × *g*, 4°C), serum was stored at -80°C until analyzed. The body condition score (BCS) of each animal was recorded at each blood sampling time using a five-point system (Edmonson et al., 1989). The mean BCS of the animals was 3.1. Biopsies were collected from s.c. fat at the tail head and from liver tissue at days -21, 1, 21, 70, 105, 182, 196, 224 and 252 relative to parturition with the following procedure: after local anesthesia (Procaine 2%, CP-Pharma, Burgdorf, Germany) using Tru-Cut® biopsy needles (Traveno Laboratories Inc, IL, USA, Ø 2.2 mm), liver was transcutaneously biopsied under ultrasound control using an automatic device (BARD®, Tempe, AZ). For the biopsies from s.c. AT, the skin adjacent to the tail head was incised (2 cm) with a scalpel under sacrococcygeal epidural anesthesia (6 ml 2% Procaine) at the contralateral side of the preceding sampling. Adipose tissue was then excised, frozen in liquid nitrogen, and stored at -80°C. The skin wounds were treated with a ceftiofur hydrochloride antibiotic (Excenel®, Pharmacia, Erlangen, Germany) and closed.

2.1.2. Trial 2

German Holstein-Friesian heifers (half siblings, all raised under the same conditions, n = 25) with an average age at first calving of 23 ± 0.2 months and a mean

BCS of 3.0 were used. The study is described in detail by von Soosten et al. (2011). Before calving the heifers were kept in group pens and were fed *ad libitum* with a PMR (60% corn silage and 40% grass silage (6.7 MJ NE_L/kg DM) and 2 kg concentrate (6.7 MJ NE_L/kg DM per day). The PMR fed postpartum (13.5 MJ NE_L/kg DM) comprised 25% grass silage, 38% corn silage and 37% concentrate on a DM basis. In addition, 4 kg concentrate containing the fat supplements were fed daily. On the day of parturition, 5 animals were slaughtered for determining body composition, the weights of various organs and of s.c. and v.c. fat depots and for sample collection. The remaining animals were shifted to the postpartum diet and were randomly allocated to either CLA supplementation (100 g/day, n = 10) or CTR fat supplement (CTR, n = 10). Supplemental fats were as described for trial 1. The animals of the CLA group consumed 6.0 g/day of the *trans*-10, *cis*-12 and 5.7 g/day *cis*-9, *trans*-11 of CLA isomer. Five animals of each group were then sacrificed on day 42 and 105 postpartum. Blood samples were collected on days -21, -14, -7, -3, 1 (n = 25), 7, 14, 28, 42 (n = 20) and 105 (n = 10) relative to parturition. At slaughter, fat samples were collected from 3 different v.c. depots (omental, mesenterial and retroperitoneal) and 3 s.c. depots (beside the tail head, above withers and sternum) and from liver tissue. The samples were snap-frozen in liquid nitrogen and stored at -80°C; for immunohistochemistry the samples were immediately fixed in 4% paraformaldehyde overnight.

2.2. Determination of serum Hp, NEFA and ß-hydroxybutyrate concentrations

An ELISA as described by Hiss et al. (2009) was used. The serum used as standard had been calibrated against a standard obtained from a 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. The intra- and interassay coefficients of variation were 3.9% and 12.2%, respectively.

Blood samples were analyzed for NEFA and ß-hydroxybutyrate concentrations with commercial kits (NEFA-C, Wako Chemicals GmbH, Neuss and RANBUT, Randox Laboratories GmbH, Wülfrath, Germany).

2.3. Immunohistochemistry for Hp in AT

The tissue samples from trial 2 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 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) and finally dried at 60°C for 2 h and thereafter at 37°C overnight. Sections were deparaffinized in Roti®-Histol, then gradually rehydrated in descending grades of isopropanol (100%, 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 three times for each 5 min at 700 W, and then the sections were allowed to cool at room temperature for 30 min and washed with distilled water. Elimination of endogenous peroxidase activity was done using 3% H_2O_2 for 15 min, the sections were washed three times for each 5 min with PBS (pH 7.2, 0.05% Tween 20), and 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) 1:2000, in PBS for 15 h. The sections were subsequently incubated with goat anti-rabbit IgG coupled with horseradish peroxidase (HRP; 1:200 in PBS, Southern Biotech, Birmingham, AL) 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 washing with tap water for 10 min, the sections were mounted with Kaiser's glycerol gelatine (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 200 x magnification and photographed with a digital camera (JVC, Hachioji Plant of Victor Company, Tokyo, Japan).

2.4. Quantification of Hp mRNA abundance in tissues

Liver and s.c. fat biopsies from CTR cows at days -21, 1, 21, 70, 105, 182, 196, 224, and 252 and from CLA cows, at days -21, 21, 105, 196 and 252 were homogenized

using liquid nitrogen, mortar and pestle in case of samples from CLA cows receiving 100 g CLA/day from trial 1, and using the Precellys®24 system (peQLab Biotechnology, Erlangen, Germany) for all the samples from trial 2. Total RNA was extracted from the homogenates using Trizol (Invitrogen, Karlsruhe, Germany) and purified using spin columns (RNeasy® Mini Kit, Qiagen, Hilden, Germany) after DNase (Qiagen) digestion in solution. The concentration of RNA and the purity were controlled by absorbance readings at 260 nm and 280 nm using the Nanodrop 1000 (peQLab Biotechnology). The integrity of the RNA was assessed using ethidium bromide denaturing RNA gel electrophoresis and rechecked in selected samples from each tissue by microcapillary electrophoresis using the Bioanalyzer 2100 and RNA 6000 Nano Kit system (Agilent, Waldbronn, Germany) to determine RNA Integrity Number (RIN = 7.27 ± 0.12). For cDNA synthesis, a reverse transcription of 250 ng total RNA per 20 µL reaction volume was performed with RevertAid™ reverse transcriptase (Fermentas, St Leon-Rot, Germany) according to the manufacturer's instructions, except that only 1 µL of dNTP Mix (10 mM of each dNTP, Fermentas) was used, with a Multicycler PTC 200 (MJ Research, Watertown, Mass.). Each run comprised a negative template control and a no reverse transcriptase control. Inter run calibrator was prepared using aliquots from each cDNA reaction per trial separately. To reduce the variation in cDNA synthesis, reverse transcription was performed in duplicates from each tissue which were then combined for quantitative PCR. Selection of the most stable reference genes (REF) was done out of 7 different REF following a similar procedure as described by Hosseini et al. (2010); for trial 1, Glyceraldehyde-phosphate-dehydrogenase (GAPDH), Lipoprotein receptor-related protein 10 (LRP10) and RNA polymerase II (POLR2A) and Eucariotic translation initiation factor 3 (EIF3K), LRP10, POLR2A were selected for s.c. fat and liver, respectively, using the geNorm program (Vandesompele et al., 2002). Triplicates with 2 µL cDNA (diluted 1:4) as template and 5 µL SYBR Green Jump Start Taq Readymix (Sigma-Aldrich, Steinheim, Germany) in a total volume of 10 µL were run in an Mx3000P cycler (Stratagene, Amsterdam, Netherlands).

For the samples from trial 2, 7 REF [EIF3K, LRP10, POLR2A, GAPDH, Emerin (EMD), Marvel domain containing 1 (MARVELD1) and Hippocalcin-like 1 (HPCAL1)] were measured in all samples and the most stable set of REF was selected for normalization. Co-regulation between the REF was evaluated using the ingenuity pathway analysis (IPA, Redwood City, CA). The REF used for the different tissues or tissue sets are summarized together with the time-temperature conditions and primers

characteristics in Table 1. The reaction efficiency for each gene and tissue is listed in Table 2. All reactions contained a negative template control for quantitative PCR, a negative template control and no reverse transcriptase control of cDNA. Quantification of the cycle values was converted to relative values based on the efficiency calculated per gene out of standard 1 to 10 serial dilutions of PCR amplicons with known concentrations. Quantities were normalized based on the geometric mean of REF and then calibrated based on the inter-run calibration proposed by Hellemans et al. (2007).

2.5. Statistical Analyses

All statistical analyses were performed using SPSS (version 17.0, SPSS Inc., Chicago, IL). For trial 1, the mixed model procedure was used each for serum Hp and Hp mRNA as dependent variable. Treatment (CTR, CLA-50 and CLA-100) was considered as fixed factor, and sampling dates (time) as a repeated effect, and the respective interaction was included into the model. Any clinical observations (only classified as positive or negative findings) were considered in the model as random effect to take disease specific variations between groups into account. For serum Hp, but not for mRNA analyses which were limited to the cows from trial 1, parity (cows or heifers) was considered as fixed effect together with its interaction with treatment. The covariance structure heterogeneous first-order autoregressive and Bonferroni correction were used for serum data, while component symmetry was used for mRNA data.

For trial 2 in which the repeated design was not applicable, all data were tested for homogeneity of variances ($P \leq 0.1$). With regard to the homogeneity of variances, differences between CTR and CLA group were pairwise compared by Students´ t-test ($P \leq 0.05$; each time point for serum Hp concentrations and mRNA data). In a GLM model, clinical observations (diseased or not diseased) were considered as random effect to test for possible disease specific variations between groups. Correlations were calculated using Pearson correlation coefficient at $P \leq 0.05$; reported correlations herein are limited to $r > 0.4$. All data are presented as mean ± SEM.

3. Results

3.1. Serum Hp concentrations

The time dependent changes of the serum Hp concentrations from the entire

experimental period (day -21 to day 252 of lactation) in trial 1 are shown in Fig. 1 for both heifers and cows. During the first week after calving, Hp concentrations were increased compared to the preceding values, then decreased again and remained relatively constant thereafter until the end of the observation period. When considering all animals during the time relevant for CLA treatment, i.e., excluding the post treatment and prepartum interval, only sampling time, parity (cows versus heifers) and the interaction between parity and treatment were significant. These findings were confirmed when considering heifers and cows separately and were also observed in trial 2 (data not shown).

When testing for potential relationships between serum Hp values and estimates of body fat (BCS), recordings of body weight, fat mass and body fat portion, no correlations were neither observed during the entire experimental period nor at certain dates. For NEFA and ß-hydroxybutyrate, indicators of lipolysis and production of ketone bodies, quantified in the serum samples of both trials (Pappritz et al., 2011; von Soosten et al., 2011), moderate correlations were observed, in particular when limiting the data to the time during which the probability for metabolic stress is highest (days -21 to 49 postpartum): the correlations ($P < 0.01$) between the serum Hp concentrations and NEFA in primiparous cows were $r = 0.47$ in trial 1 and $r = 0.54$ in trial 2. For the pluriparous cows from trial 1, the association was less strong ($r = 0.24$); the correlations between Hp and ß-hydroxybutyrate were consistently below $r = 0.4$.

3.2. Haptoglobin mRNA abundance in liver and AT

In trial 1, haptoglobin mRNA abundance in AT was considerably lower ($P < 0.05$) than in liver. In comparison to s.c. and v.c. fat depots, Hp mRNA abundance in liver was 201 and 602 fold greater in trial 2. In about 50% of the AT biopsies from s.c. tail head fat collected in trial 1, Hp mRNA was below the limit of detection of the qPCR system. When assessing the relationship between serum Hp concentrations and Hp mRNA abundance in liver, positive correlations ($P < 0.001$) were established for both the biopsies (trial 1; $r = 0.65$) and the post mortem samples (trial 2; $r = 0.67$).

In biopsies collected from the cows in trial 1, time dependent changes ($P < 0.05$) were observed for Hp mRNA abundance reaching greatest values in liver at the day of calving and on day 21 in s.c. fat, respectively (Fig. 2). Two weeks after the end of the CLA supplementation period, Hp mRNA abundance in liver exceeded the values

recorded during the preceding supplementation period reaching the level of significance when compared against day 21. However, this increase after the supplementation period was also visible in the animals receiving the CTR fat supplement. In contrast, Hp mRNA abundance in AT biopsies remained at a relatively constant level after day 21 throughout the further observation period.

For the liver samples collected in trial 2 after the animals were killed, the greatest Hp mRNA abundance was seen on day 1 after calving (Fig. 3); the values obtained at days 42 and 105 of lactation were about 8-fold less than this initial value. Similar time dependent patterns were obtained for AT samples collected from 3 different s.c. and 3 v.c. depots (Fig. 4), however, in mesenterial fat, the time effect was insignificant; in s.c. fat from the sternum area, the time difference was limited to day 1 versus day 105. Treatment with CLA yielded no significant effects on Hp mRNA abundance in the different fat depots, with the exception of omental fat and s.c. fat from the withers area in which Hp mRNA abundance was lower ($P < 0.05$) in the CLA supplemented heifers than in the CTR animals on days 42 and 105. The Hp mRNA abundance in the s.c. fat depots was generally higher than in the v.c. depots ($P < 0.05$). Subcutaneous fat from the tail head had the greatest and mesenterial fat had the lowest values. Haptoglobin mRNA abundance in the different tissues from trial 2 was correlated, whereby all s.c. depots were interrelated. In contrast, significant correlations ($P < 0.01$) between the v.c. depots were limited to mesenterial and retroperitoneal fat. Each s.c. fat depot was correlated to mesenterial and retroperitoneal fat, but for omental fat, there was a moderate correlation only with tail head fat. Correlations between liver and fat Hp mRNA abundance were limited to omental fat and s.c. fat from withers area.

3.3. Immunohistochemical detection of the Hp protein in different fat depots

Haptoglobin immune reactive staining was detectable in both the lymph node sections used as positive controls and in all s.c. and v.c. fat depots. The localization and staining intensity in the different fat depots is exemplified in Figure 5. Haptoglobin immunoreactivity was limited to the adipocytes, whereas the cells of the stromal-vascular fraction were all negative. Within the adipocytes, Hp immune signals were observed along the cytoplasmic rim surrounding the large fat droplet.

4. Discussion

4.1. Haptoglobin as an adipokine in cattle

The evidence of Hp mRNA expression in AT and the presence of Hp-immuno staining in histological sections from different fat depots confirm that this APP can be classified as an adipokine in the bovine species, too. Based on the results from immunohistochemistry, the expression of Hp is likely attributable to the adipocytes. Moreover, when using cultivated adipocytes derived from isolated primary bovine preadipocytes as described by Hosseini et al. (2011), we were able to qualitatively detect Hp mRNA both in undifferentiated and differentiated adipocytes (Saremi et al., 2010). In humans, different Hp mRNA abundances in different fat depots have previously been reported whereby epicardial fat had 48 to 111-fold higher values than 3 different s.c. depots but only 1.6-fold higher Hp mRNA expression than omental fat (Fain et al., 2010). When ranking the different bovine depots from our study according to their Hp mRNA abundance values across all sampling dates, we did not observe a difference between the 3 s.c. depots and omental fat, but the Hp mRNA abundance in s.c. depots was higher than in retroperitoneal and mesenterial fat.

Like other inflammatory biomarkers, Hp has been suggested as being linked to obesity, insulin resistance, and the metabolic syndrome in humans. Based on the associations between body fat and serum Hp and on the evidence of Hp mRNA and protein in biopsies from s.c. abdominal fat as well as the release of Hp from s.c. and v.c. human AT explants (Fain et al., 2004) from obese subjects, a contribution of body fat to circulating Hp concentrations was concluded and Hp was proposed as adiposity marker in humans (Chiellini et al., 2004). In view of the low Hp mRNA abundance we observed in AT as compared to liver, we speculate that AT borne Hp will not significantly contribute to Hp serum concentrations. Due to the fact that we have no quantitative data on Hp protein concentration in AT, we did a rough estimation from our mRNA data to give an idea about the quantitative relationships which is based on three assumptions (1) mRNA concentration would be directly corresponding to protein secretion, (2) concentrations of total RNA per g of tissue would parallel the yields of extraction achieved in fat and liver tissue and (3) expression within the fat depots and within the entire liver would be homogenous. Doing so we took the tissue masses (von Soosten et al., 2011) and mRNA yields per g of tissue into consideration. Accordingly, the relative contribution of v.c. fat would be 2.8 fold greater compared to s.c. fat. In comparison to liver, the Hp production

from all v.c. and s.c. depots would amount to only 0.02% of the hepatic one.

In our studies, we did not see any relation between serum Hp and BCS, body mass and fat portion in late pregnant or lactating heifers and cows. The question as to whether Hp in serum might increase in cattle if fat mass exceeds a certain level remains yet unresolved but appears to be of minor relevance for dairy cows anyway; the animals enrolled in our studies were definitely neither obese nor overconditioned. Nevertheless, the lack of an evident relationship between circulating Hp concentrations and normal body fat portions in dairy cattle demonstrates that variation in the degree of adiposity does not itself influence serum Hp concentrations and thus does not confound the use of Hp as an inflammatory marker. The correlations observed between serum Hp and NEFA point to the relation between the development of fatty liver and induction of Hp. An increased release of NEFA from adipose tissue knowingly might exceed the liver's capacity for fatty acid oxidation and discharge into circulation thus leading to fatty liver (Katoh, 2002), which might induce increased Hp concentrations.

4.2. Physiological changes of serum Hp concentrations and Hp mRNA abundance in liver and fat throughout lactation

The longitudinal characterization of the serum Hp concentrations throughout lactation as done in trial 1 indicates that apparently physiological changes seem to be limited to parity, parturition and, to a lesser extent, late lactation. The reason for the higher Hp concentrations observed in heifers than in cows during the first week after parturition in trial 1 are not clear, but were also reported by Cullens (2005) indicating that parturition may elicit a higher acute phase reaction in primiparous than in multiparous cows. The peripartal peak in Hp concentrations observed in both trials is in accordance with several other reports in cattle (Ametaj, 2005; Humblet et al., 2006; Hachenberg et al., 2007; Tóthová et al., 2008) and is probably due to parturition-related tissue lesions and inflammatory reactions of the genital tract. In addition, this increase might be influenced by metabolic stress (Katoh, 2002; Bionaz et al., 2007; Hachenberg et al., 2007). The notion of liver being the main source of circulating Hp was confirmed by the time dependent pattern of hepatic mRNA abundance in both trials being largely analogous to the serum values. In view of the low Hp mRNA abundance in fat tissues as compared to liver, a substantial contribution of AT to serum Hp seems improbable. Nevertheless, the observation of similar time dependent patterns, i.e., highest abundance

values on day 1 after calving in both liver and fat depots indicates similar regulatory pathways in both tissues. Parturition is accompanied by comprehensive endocrine changes, e.g., a rise of glucococorticoids (Hoffmann et al., 1973) and the final stages of cervical ripening and parturition have been suggested to resemble an inflammatory process with increased release of proinflammatory cytokines (van Engelen et al., 2009). Indeed, Lee et al. (2010) have recently shown that Hp gene expression was induced by the synthetic glucocorticoid dexamethasone in both s.c. and omental human fat.

4.3. Effects of supplementation with CLA on serum Hp concentrations and tissue mRNA abundance

The efficacy of the current CLA supplementation in terms of milk fat reduction has been reported earlier for both trials (Pappritz et al., 2011; von Soosten et al., 2011). Supplementation with CLA may have anti-inflammatory and/or immune-ameliorating effects (Yu et al., 2002), whereby *cis*-9, *trans*-11 CLA is supposed to be responsible for the anti-inflammatory effect attributed to CLA as recently reviewed by Reynolds and Roche (2010). In contrast, *trans*-10, *cis*-12 CLA has been demonstrated to promote inflammation in primary cultures of differentiated human adipocytes (Martinez et al., 2010). It is likely that dose, duration, and isomeric composition of CLA each impact the effects of CLA supplementation regimes. So far no studies have been published on the effects of long term CLA supplementation on inflammatory markers in ruminants. For Hp, Noto et al. (2007) showed that Zucker rats fed with mixed CLA isomers for 8 week had lower serum Hp concentrations than untreated controls. However, for another APP i.e. for C-reactive protein, increased concentrations were reported in nonobese humans and in patients affected with the metabolic syndrome when supplemented with CLA as mixed isomers (*cis*-9, *trans*-11 and *trans*-10, *cis*-12) (Riserus et al., 2004; Smedman et al., 2005). In the trials we performed, serum Hp concentrations were not affected by CLA treatment. When considering Hp mRNA abundance in liver, again no effect of the CLA supplementation could be identified thus disagreeing with the notion that CLA isomer mixture used in this study may exert systemic anti-inflammatory effects. However, the observation of reduced Hp mRNA abundance in CLA versus CTR animals in another s.c. depot (from withers) and one v.c. depot, i.e. omental fat, may indicate a locally divergent responsiveness of AT towards CLA. In view of the lack of effects on serum Hp concentrations, the observed reaction in two out of six different fat depots seems of

marginal importance for the organisms as an entity.

5. Conclusions

Haptoglobin mRNA and protein expression in adipose tissue qualified this acute phase protein as adipokine in cattle. The differences in Hp mRNA abundance between different subcutaneous and visceral depots indicate that biopsies from subcutaneous depot are not necessarily representative when aiming to characterize adipose regulation. Dietary CLA supplementation had no effect on serum Hp and thus anti-inflammatory effects of the CLA supplementation protocol used herein are not supported. However, the function of Hp expression in AT for both local and systemic homeostatic or homeorhetic adaptations in lactating dairy cows remains to be elucidated.

Conflict of interest statement

None of the authors of this paper has a financial and personal relationship with other people or organizations that could inappropriately influence or bias the content of the paper.

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Table 1 (A): List of the tissue specific sets of reference genes identified as being most stable and thus used for normalization. (B): Characteristics of the primers and the real-time PCR conditions

(A):

Individual or combined tissues	Reference genes used
Omental and retroperitoneal adipose tissues (AT)	EIF3K, LRP10, POLR2A, EMD, MARVELD1
Liver	EIF3K, LRP10, POLR2A, GAPDH, HPCAL1
Individual tissues:	
- Mesenterial AT	
- Each s.c. AT (Tail head, Withers, Sternum)	EIF3K, LRP10, POLR2A, EMD, MARVELD1, HPCAL1
Combinations of:	
All AT depots	
All AT depots and liver*	EIF3K, LRP10, POLR2A, HPCAL1

(B):

Primers	Sequence (5'-3')	NIH Genbank accession number	bp[9]	Amount (nmol)	Mean Cq[10] value[11]	Annealing[12] (sec/˚C)
Haptoglobin[1]						
Forward	GTCTCCCAGCATAACCTCATCTC	AJ271156	174	800	25.37	30/58
Reverse	AACCACCTTCTCCACCTCTACAA					
EIF3K[2]						
Forward	CCAGGCCCACCAAGAAGAA	NM_001034489	125	400	22.87	45/59
Reverse	TTATACCTTCCAGGAGGTCCATGT					
LRP10[3]						
Forward	CCAGAGGATGAGGACGATGT	Bc149232	139	400	21.90	30/61
Reverse	ATAGGGTTGCTGTCCCTGTG					
POLR2A[4]						
Forward	GAAGGGGGAGAGACAAACTG	X63564	86	800	23.86	60/60
Reverse	GGGAGGAAGAAGAAAAAGGG					
GAPDH[5]						
Forward	AATGGAAAGGCCATCACCATC	U85042	204	400	19.02	75/59
Reverse	GTGGTTCACGCCCATCACA					
EMD[6]						
Forward	GCCCTCAGCTTCACTCTCAGA	NM_203361	100	400	22.07	45/59
Reverse	GAGGCGTTCCCGATCCTT					
MARVELD1[7]						
Forward	GGCCAGCTGTAAGATCATCACA	NM_001101262	100	400	22.61	45/59
Reverse	TCTGATCACAGACAGAGCACCAT					
HPCAL1[8]						
Forward	CCATCGACTTCAGGGAGTTC	NM_001098964	99	400	23.98	30/60
Reverse	CGTCGAGGTCATACATGCTG					

[1]Hiss et al., 2004.
[2]EIF3K: Eucariotic translation initiation factor 3, subunit K (Kadegowda et al., 2009).
[3]LRP10: Lipoprotein receptor-related protein 10 (Hosseini et al., 2010).
[4]POLR2A: RNA polymerase II (Hosseini et al., 2010).
[5]GAPDH: Glyceraldehyde-phosphate-dehydrogenase (Hosseini et al., 2010).
[6]EMD: Emerin (Kadegowda et al., 2009).
[7]MARVELD1: Marvel domain containing 1 (Kadegowda et al., 2009).
[8]HPCAL1: Hippocalcin-like 1 (Hosseini et al., 2010).
[9]bp: base pairs.
[10]Cq: Quantification cycle.
[11]Based on post mortem samples from trail 2.
[12]Initial denaturation (min/˚C) 10/95, Denaturation (sec/˚C) =30/95, Extention (sec/˚C) =30/72; for RNA polymerase extention conditions were 60/72.
* For selection of stable reference genes, mammary gland (data not shown) was also included in the tissue set.

Table 2. Efficiency of real-time PCR per tissue defined by amplicon standard curves*

Genes	Trial 1		Trial 2						
	s.c. tail	liver	s.c. sternum	s.c. wither	s.c. tail	mesenterial	omental	retroperitoneal	liver
Haptoglobin	94.9	93.6	85.2	88.4	97.6	102.9	97.4	93.9	98.1
EIF3K	---	93.3	95.3	95.3	95.0	97.5	95.9	93.3	94.3
LRP10	102.4	102.4	94.2	93.5	91.1	93.9	92.3	92.6	96.1
POLR2A	90.3	92.1	92.7	97.9	93.4	97.5	96.4	97.5	93.5
GAPDH	84.7	---	82.8	93.7	91.4	90.0	89.5	89.2	91.0
EMD	---	---	97.1	96.5	96.6	94.2	94.2	97.4	97.6
MARVELD1	---	---	95.0	97.7	94.0	90.3	98.3	96.1	97.0
HPCAL1	---	---	92.3	89.6	91.3	86.5	87.4	90	92.2

*Standard curves consist of 5 point 10-fold dilution series.

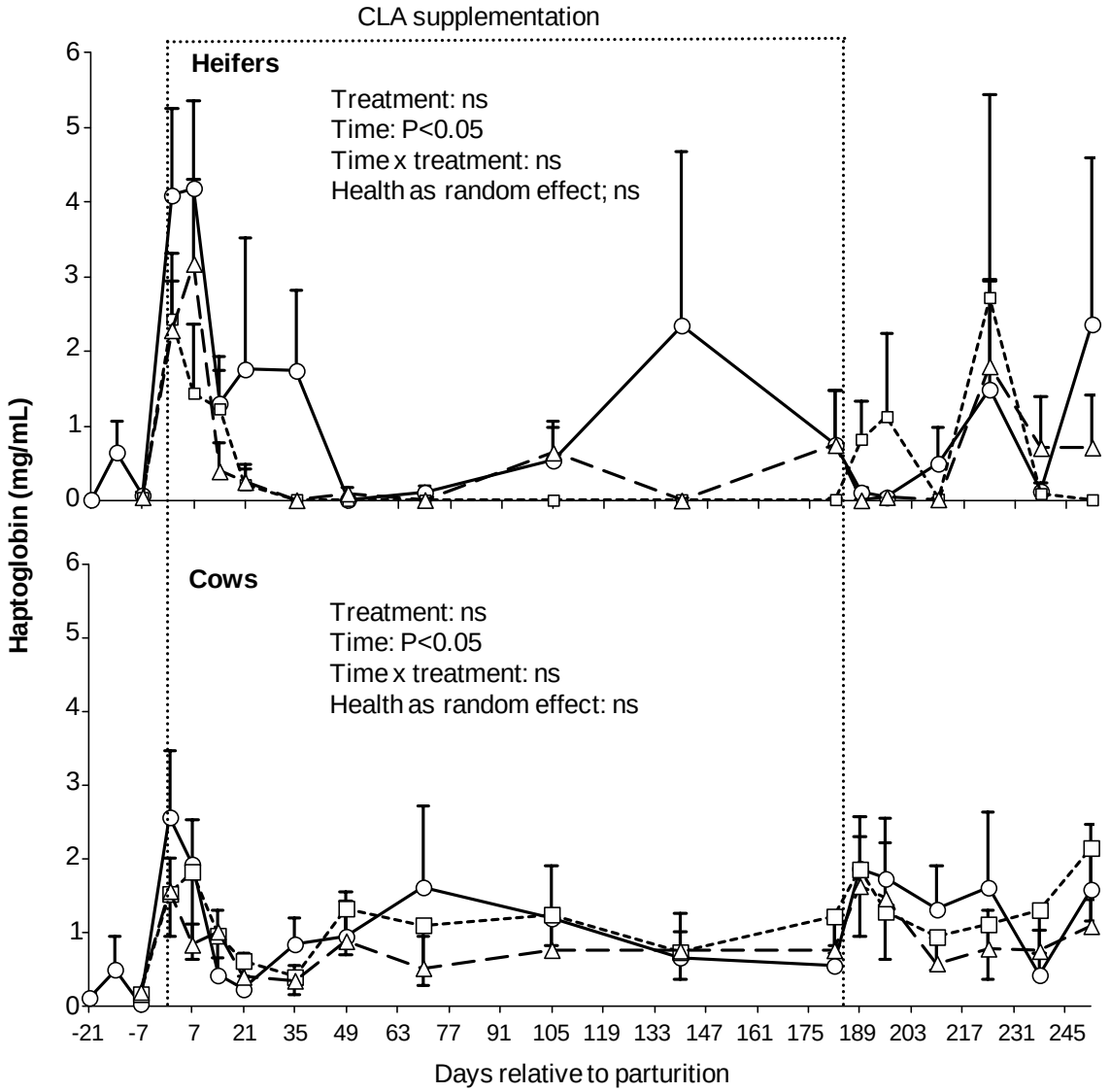

Fig. 1. Serum haptoglobin concentrations (means ± SEM) in cows and heifers receiving conjugated linoleic acids (CLA, Lutrell® Pure, BASF SE, Ludwigshafen, Germany) at 50 or 100 g/day or a control fat supplement (Silafat®, BASF SE) from day 1 until day 182 postpartum in trial 1. The statistical results included in the graph comprise the treatment and post treatment period (day 1 to 252); all animals and samples were included in the figure. The concentrations of haptoglobin in cows versus heifers were different (P<0.05). [Control (○): cows n = 11, heifers n = 6; CLA-50 (□): cows n = 11, heifers n = 5; CLA-100 (Δ): cows n = 11, heifers n = 5]. n: number of animals. ns: not significant.

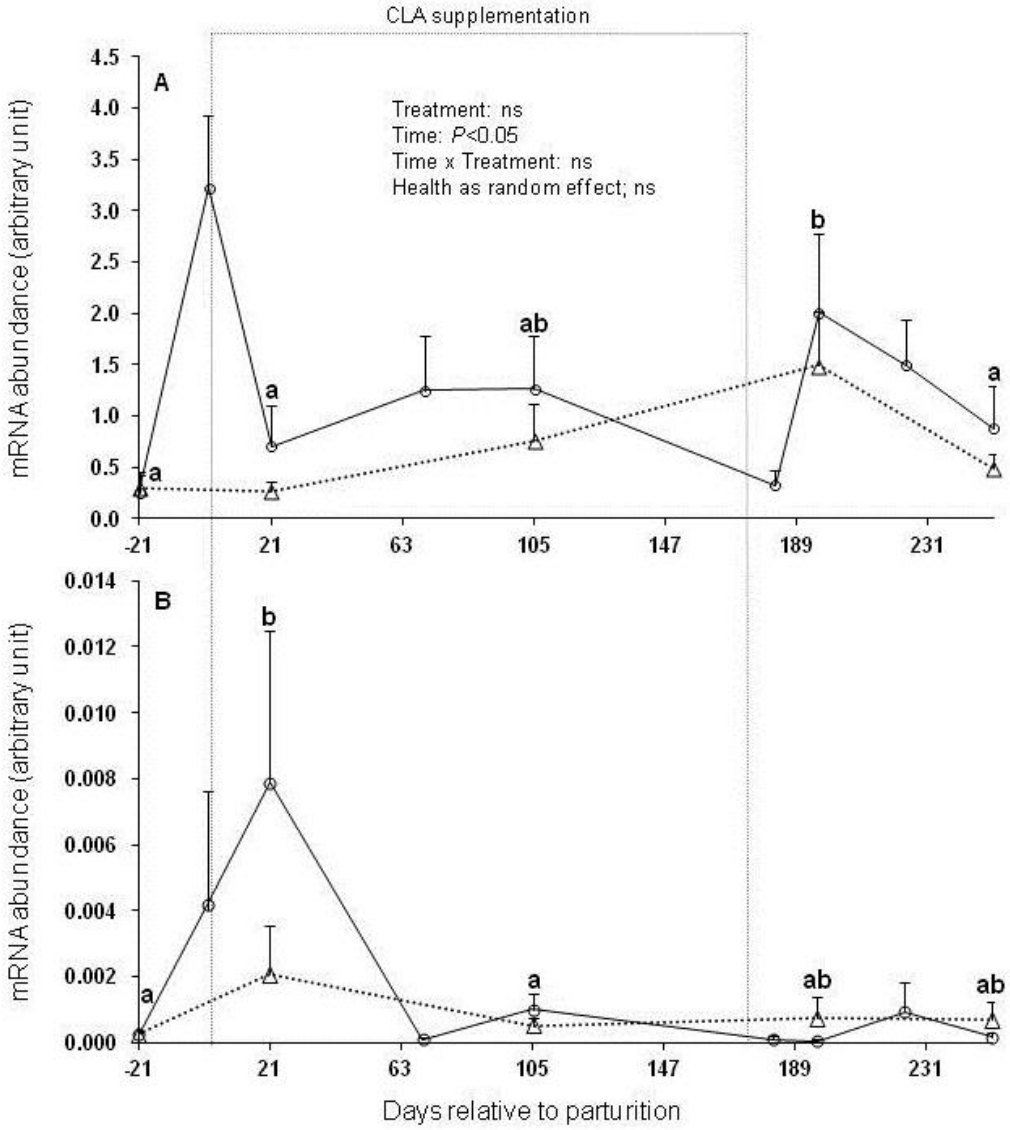

Fig. 2. Haptoglobin mRNA abundance (means ± SEM) in liver (A) and in subcutaneous tail head fat (B) tissue in cows receiving conjugated linoleic acids (CLA, Lutrell® Pure, BASF SE, Ludwigshafen, Germany) at 100 g/day CLA or a control fat supplement (Silafat®, BASF SE) from day 1 until day 182 postpartum in trial 1. Samples for mRNA analyses were limited to cows thus excluding heifers and to the CLA dose of 100g/d thus excluding the 50g CLA/d group. Samples from days 1, 70, 182, and 224 were also restricted to the control cows. [Control (○): n = 11, CLA-100 (Δ): n = 11]. Different letters indicate significant (P<0.05) differences between the different days. ns: not significant. Data are normalized based on the geometric mean of Glyceraldehyde-phosphate-dehydrogenase (GAPDH), Lipoprotein receptor-related protein 10 (LRP10) and RNA polymerase II (POLR2A) for subcutaneous tail head fat and Eucariotic translation initiation factor 3 (EIF3K), LRP10, and POLR2A for liver.

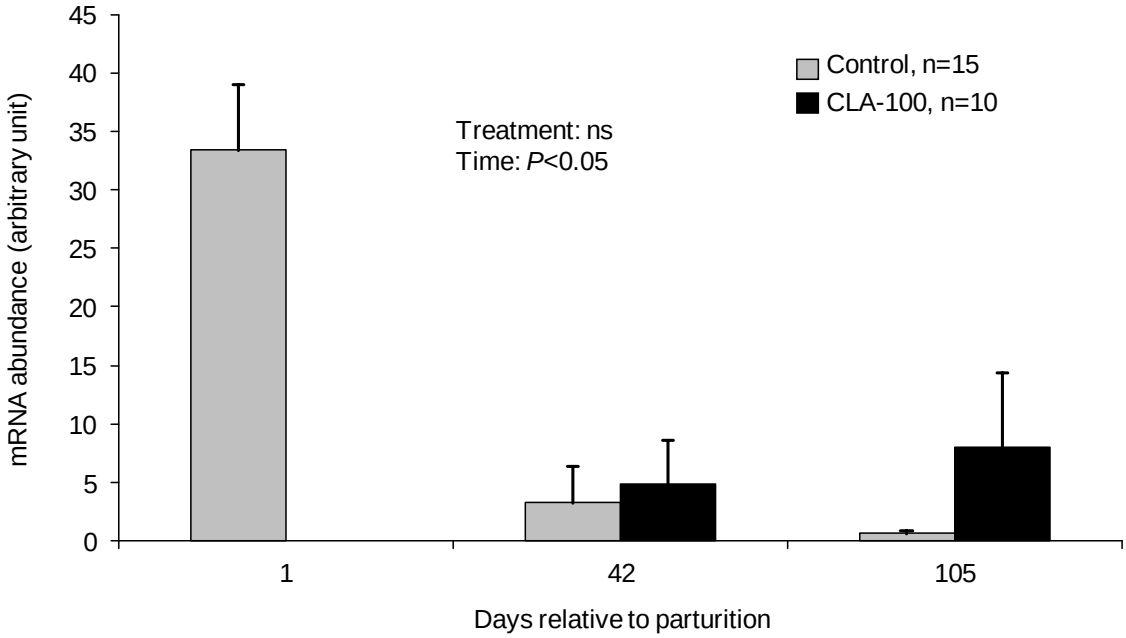

Fig. 3. Haptoglobin mRNA abundance (means ± SEM) in liver tissue of heifers receiving conjugated linoleic acids (CLA, Lutrell® Pure, BASF SE, Ludwigshafen, Germany) at 100 g/day or a control fat supplement (Silafat®, BASF SE) from day 1 until day 105 postpartum in trial 2. Significant time effect is between day 1 and day 42 and 105, respectively. Health as random effect was insignificant. ns: not significant. Data are normalized based on the geometric mean of the reference genes listed in table 1.

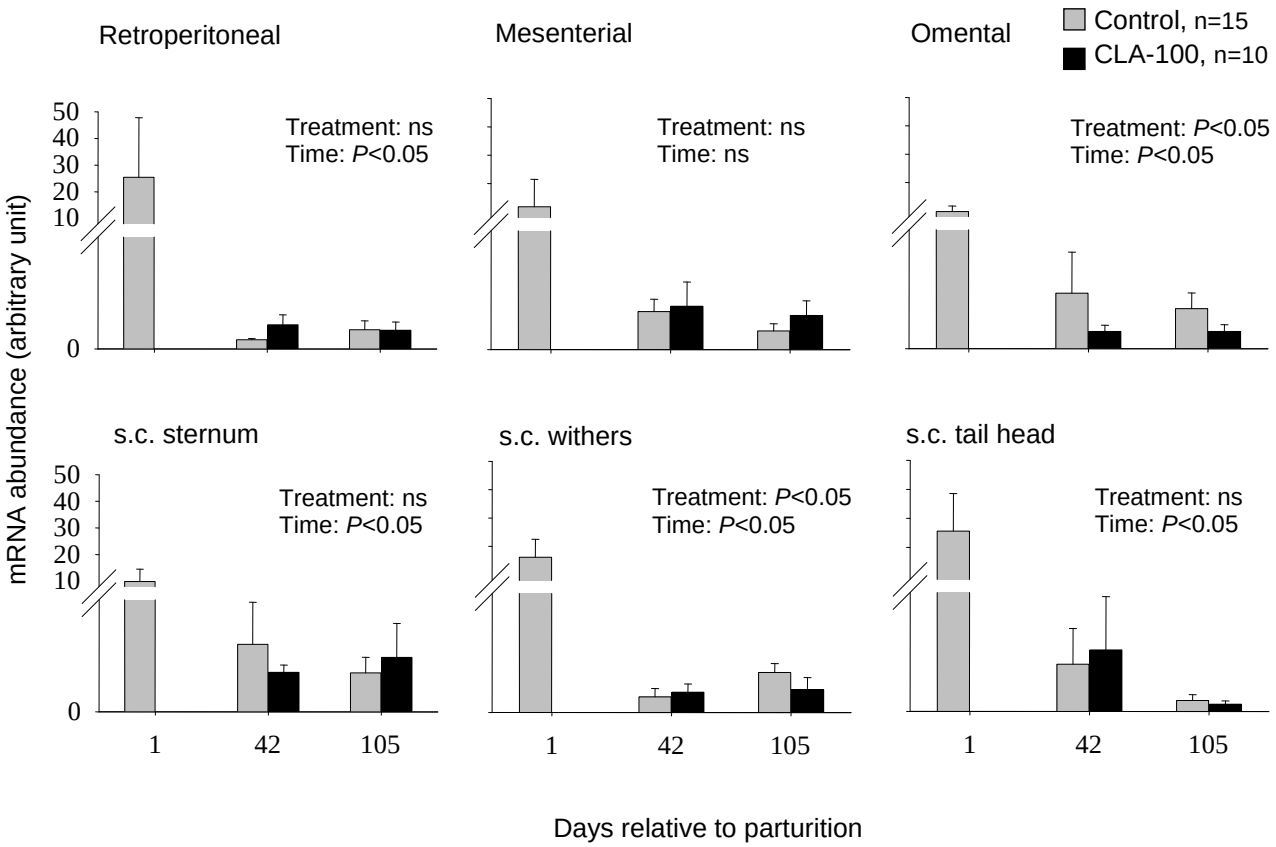

Fig. 4. Haptoglobin (Hp) mRNA abundance (means ± SEM) in three visceral (upper panels) and three subcutaneous (s.c.) fat depots (lower panels) in heifers supplemented with 100 g/day conjugated linoleic acids (CLA, Lutrell® Pure, BASF SE, fLudwigshafen, Germany) or a control fat supplement (Silafat®, BASF SE) from day 1 until day 105 postpartum. On day 1, Hp mRNA abundance was higher (P<0.05) than on day 42 and on day 105; only in s.c. fat from sternum the difference was limited to day 1 versus day 105. Health as random effect was insignificant. The effects of CLA treatment were limited to s.c. fat from withers and omental v.c. fat in which reduced Hp mRNA abundance was observed. ns: not significant. Data are normalized based on the geometric mean of the reference genes listed in table 1.

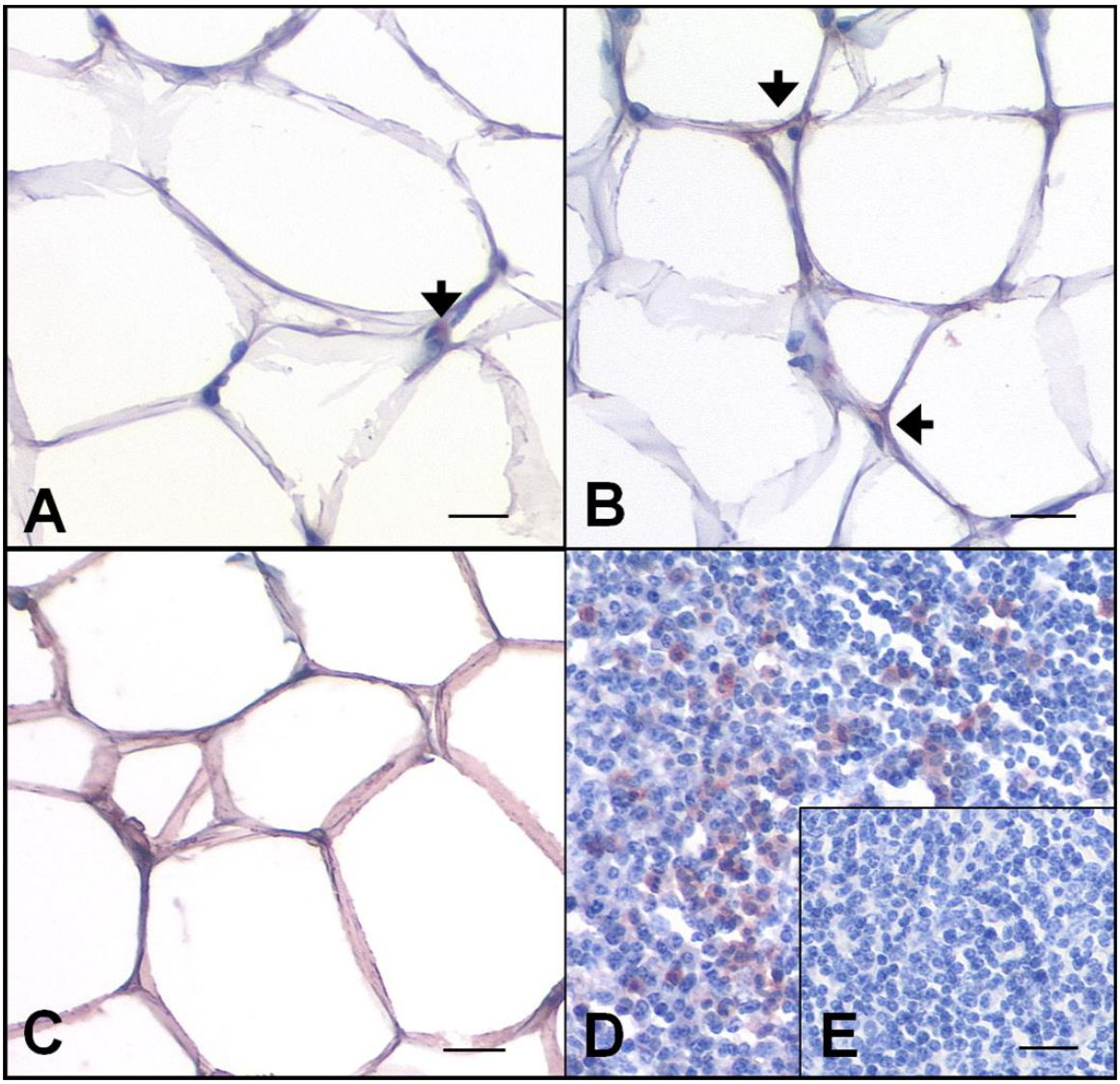

Fig. 5. Examples of haptoglobin immunoreactivity in histological sections from different bovine adipose tissues (A, B and C) and lymph nodes (D, E). Representative positive cells appear as red staining in the cytoplasm near adipocyte nuclei (marked with arrow heads) of subcutaneous sternum (A) and tail head (B) fat depots as well as along the whole cytoplasmic rim of mature adipocytes from mesenteric fat depots (C). Bovine lymph nodes were used as positive (D) and negative (E) controls. Original magnification: 200-fold. Scale bars represent 20 µm.

5. Manuscript 3 (submitted)

Hepatic and extrahepatic expression of serum amyloid A3 during lactation in dairy cows

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ABSTRACT

Serum amyloid A3 (SAA3) is the predominant SAA isoform secreted by mammary epithelial cells in dairy cows; it is also expressed in bovine adipose tissue (AT). The adipokine SAA3 is linked to obesity and insulin resistance of AT and the respective inflammatory response, at least in mice. Dietary treatment with conjugated linoleic acids (CLA) reportedly affects also insulin sensitivity and inflammatory status in monogastrics. Both SAA3 and CLA thus seem to alter similar functions. Based on changes in insulin sensitivity and the inflammatory status throughout lactation, we hypothesized that the mRNA abundance of *SAA3* in various tissues might be regulated as well and that CLA could be a modulator of *SAA3* mRNA expression. In two trials, 21 pluriparous and 25 primiparous Holstein cows were fed with 100 g/d of a CLA or a control fat supplement from d 1 until d 182 or d 105 postpartum (p.p.), respectively. Biopsies from liver and

subcutaneous (s.c.) AT from pluriparous cows and samples from 3 different visceral (v.c.) AT and 3 s.c. AT, muscle, mammary gland, and liver tissue from slaughtered primiparous cows were obtained. In an adipocyte cell culture system, cell samples were collected during differentiation of bovine preadipocytes at d 0, 2, 6, 8, 10, 12, and 13 relative to the onset of differentiation. The *SAA3* mRNA abundance in tissues and in differentiating bovine preadipocytes was measured by real-time PCR. The presence of the SAA protein was confirmed by Western blotting. Treatment with CLA yielded only few and inconsistent effects on *SAA3* mRNA abundance. In both trials, *SAA3* mRNA peaked at d 1 p.p. in all tissues except in mesenteric AT in which the change was not significant. The highest *SAA3* mRNA expression was observed in the mammary gland, followed by omental AT. The SAA protein was present in the v.c. and s.c. AT depots investigated. Adipocytes as one source of *SAA3* were confirmed by the *SAA3* mRNA profile in differentiating adipocytes. The longitudinal changes observed point to *SAA3* being involved in the inflammatory situation around parturition.

Key words: *SAA3*, CLA, adipose tissue, dairy cow.

INTRODUCTION

Serum amyloid A (**SAA**) is a major acute phase protein in cattle and has proved as diagnostic marker (Eckersall and Bell, 2010). The biological functions identified until now include cholesterol transport as well as modulation of immune reaction (Ceciliani et al., 2012). Acute-phase SAA comprises two closely related isoforms, SAA1 and SAA2, which are expressed mainly in the liver in response to proinflammatory stimuli; further members of the SAA family are SAA3 and SAA4, whereby SAA3 is mainly extrahepatically expressed under proinflammatory conditions in several mammalian species (Uhlar and Whitehead, 1999). The isoform SAA4 is constitutively expressed and responds only moderately to inflammatory stimuli (de Beer et al., 1996). Due to its synthesis and secretion from adipose tissue (**AT**), SAA3 was also classified as an adipokine (Lin et al., 2001) and the circulating concentrations of SAA correlate with obesity and insulin resistance in humans (Ebeling et al., 1999, Yang et al., 2006). However, using tandem mass spectrometry, no SAA3 protein was observed in circulating high density lipoproteins, albeit secretion of SAA3 from cultured adipocytes was confirmed. Thus the increased SAA blood concentrations in obesity and insulin resistant states result rather not from increased SAA3 secretion by AT (Chiba et al., 2009).

In cattle, SAA proteins were characterized in 1988 (Husebekk et al., 1988) and SAA3 is the predominant SAA isoform secreted by mammary gland epithelial cells in response to gram-negative (Larson et al., 2005) or gram-positive bacteria (Weber et al., 2006). Based on its mammary localization it is also termed mammary SAA3 (McDonald et al., 2001) and is assumed to protect the mammary tissue during remodeling and infection (Molenaar et al., 2009). The *SAA3* mRNA has been detected in bovine AT, but its expression is apparently not upregulated by lipopolysaccharide (Mukesh et al., 2010; Berg et al., 2011). Remarkably, SAA is regulated by nutritional changes, since caloric restrictions are associated with down-regulation of SAA, both at the mRNA and the protein level (Poitou et al., 2005). Conjugated linoleic acids (**CLA**) are compounds, which are discussed to be related to insulin sensitivity and inflammatory status of the body. The *cis*-9,*trans*-11-CLA isomer (***c*9,*t*11-CLA)** improves insulin sensitivity and reduces proinflammatory cytokines (Reynolds and Roche, 2010); the *trans*-10,*cis*-12-CLA isomer (***t*10,*c*12-CLA**) adversely promotes inflammation in human adipocytes (Martinez et al., 2010) and induces lipolysis and insulin resistance (Rubin et al., 2012). Dietary CLA supplements alter lipid metabolism: in dairy cows the *t*10,*c*12-CLA has been reported to specifically target the mammary gland reducing milk fat synthesis (Baumgard et al., 2001), whereas *c*9,*t*11-CLA had less effect (Perfield II et al., 2007). In contrast to other monogastric species, body fat mass has been shown to be largely unaffected by CLA treatment in dairy cows (von Soosten et al., 2012), however we recently observed that fat cell size in primiparous cows receiving a 1:1 mixture of *c*9,*t*11-CLA and *t*10,*c*12-CLA was decreased when compared against a control group (Akter et al., 2011).

The energy requirements for milk yield and maintenance exceed energy intake by voluntary feed intake during the first weeks of lactation, in particular in high yielding dairy cows. Prioritizing milk secretion, the insulin sensitivity of peripheral tissues other than the mammary gland is decreased and body reserves mainly from AT are mobilized and imply an increased risk for metabolic diseases, mainly ketosis and fatty liver. Reducing the energy output via milk by decreasing the milk fat content through feed supplements containing CLA may attenuate the negative energy balance during this period (Bauman et al., 2011). However, based on increasing milk yield, Hutchinson et al. (2012) observed no differences in milk energy output. In contrast, Castañeda-Gutiérrez et al. (2005) reported a dose dependent reduction of milk energy output whereby energy balance was not altered. In view of the connection between SAA3 expression and lipid metabolism, we aimed to characterize the changes of *SAA3* mRNA in AT and other

metabolically important tissues throughout lactation. In particular, we aimed to compare *SAA3* mRNA abundance in different visceral (**v.c.**) and subcutaneous (s.c.) fat depots and to qualitatively characterize the SAA protein expression in these depots. Moreover, to confirm adipocytes as a *SAA3* producing cell type of AT, we quantified *SAA3* mRNA in differentiating bovine preadipocytes. In addition, we tested for potential CLA effects on *SAA3* mRNA.

MATERIAL AND METHODS

The experiments and treatments of the animals were approved by the competent authority, the Lower Saxony state office for consumer protection and food safety (LAVES, file no. 33.11.42502-04-071/07, Oldenburg, Germany). The regulations of the German Animal Welfare Act (TierSchG) in its respective current edition were met. All animals were housed at the experimental station of the Friedrich Loeffler Institute, Federal Research Institute for Animal Health, Braunschweig, Germany.

Animal experiments

Trial 1. German Holstein pluriparous cows (n = 21) were fed *ad libitum* in the entire experimental period according to the recommendations of the German Society of Nutrition Physiology (GfE, 2001). Details and outcomes in terms of diet, performance, metabolite concentrations and milk fat fatty acid profile from this experiment are described by Pappritz et al. (2011). From d 1 postpartum (p.p.) the animals were allocated to 2 groups: a CLA and a control group (10 and 11 cows, respectively) each receiving 100 g/d fat supplement during supplementation period from d 1 to d 182 p.p. The animals of the CLA group received encapsulated CLA (Lutrell Pure, BASF, Ludwigshafen, Germany). The control group received an analogous isoenergetic fatty acid mixture (Silafat, BASF) in which CLA were substituted by stearic acid. The animals of the CLA group consumed 7.6 g/d each of *t*10,*c*12-CLA and *c*9,*t*11-CLA (calculated, based on the analyzed proportions in the concentrate) and expectedly responded with reduced milk fat content to the CLA treatment whereas energy balance was not altered during most of the supplementation period (Pappritz et al., 2011). Liver and s.c. AT from the tail head region were biopsied as described earlier (Saremi et al., 2012a) on d -21, 1, 21, 70, 105 182 196, 224, and 252 from control cows and d -21, 21, 105, 196, and 252 from CLA cows relative to parturition.

Trial 2. German Holstein primiparous cows (n = 25, average age at parturition: 23 months) were fed *ad libitum* according to the recommendations of the German Society of

Nutrition Physiology (GfE, 2001). Five animals were slaughtered 1 d p.p. and the remaining cows (n = 20) were allocated randomly to either control or CLA fat supplement similar to Trial 1 starting from d 1 p.p. until slaughter. Additional information about the diets and the main outcomes in terms of body composition is available elsewhere (von Soosten et al., 2011). Five animals per group were slaughtered p.p. at d 42 and 105. Samples from 3 v.c. AT (omental, mesenteric and retroperitoneal), 3 s.c. AT (tail head, withers and sternum), liver, *M. semitendinosus*, pancreas, and mammary gland were collected immediately after slaughter. In general, efficacy of the CLA treatment in terms of milk fat reduction was demonstrated but the calculated energy balance was not significantly altered by CLA treatment. Neither NEFA serum concentrations nor BCS indicated an effect of the CLA treatment on lipolysis and body fat. However, back fat thickness was decreased in the CLA group (d 42 to 105 interval; von Soosten et al., 2011) and adipocyte sizes were decreased in 2 and 4 out of 6 different fat depots on d 42 and d 105, respectively (Akter et al., 2011).

Culture and differentiation of bovine preadipocytes

In a separate experiment, s.c. AT around sternum was obtained from 3 additional Holstein dairy cows at slaughter. Tissue treatment, cell separation, culture, and differentiation conditions are described in detail by Hosseini et al. (2012). Isolated stromal vascular cells of the 3 cows were pooled and differentiated. Cell samples of preadipocytes were taken at d 0, 2, 6, 8, 10, 12, and 13 relative to the onset of differentiation. Primary cell samples from the given d of differentiation were assayed in triplicates.

The samples from both trials were snap-frozen in liquid nitrogen. The samples from the cell culture experiment were preserved in Trizol (Invitrogen, Karlsruhe, Germany). All samples were stored at -80 °C until further use.

RNA extraction, cDNA synthesis, and relative quantification by real-time PCR

Handling of samples in order to quantify mRNA using real-time PCR was described in detail earlier (Saremi et al., 2012a; Saremi et al., 2012b). The *SAA3* primers (forward: GGGCATCATTTTCTGCTTCCT and reverse: TGGTAAGCTCTCCACATGTCTTTAG) were as described by Mukesh et al. (2010) and the primer amount used was 8 pmol per reaction. PCR conditions were 10 min at 95 °C for initial denaturation, 30 s at 95 °C for denaturation, 60 s at 60 °C for annealing, and 30 s at 72 °C for extension. A PCR amplicon standard curve was used to estimate PCR efficiency per each real-time PCR run. Two µL cDNA (diluted 1 to 4) as template and 5 µL SYBR Green JumpStart Taq Readymix (Sigma-Aldrich, Steinheim, Germany) in a total volume of 10 µL were run in an Mx3000P

real-time PCR instrument (Agilent, Santa Clara, CA). For normalization of the efficiency corrected data, reference genes from the following panel were selected and used, depending on the trial and the tissues in comparison (Saremi et al., 2012a, 2012b). For Trial 1, *GAPDH*, lipoprotein receptor related protein 10 (*LRP10*), RNA polymerase II (*POLR2A*) and eucariotic translation initiation factor 3 (*EIF3K*), *LRP10*, *POLR2A* were selected for s.c. fat and liver, respectively. For Trial 2, *EIF3K*, *LRP10*, *POLR2A*, Emerin, Marvel domain containing 1 and Hippocalcin-like 1 were used. The data used for statistics was generated by geNorm^{plus} as a part of qBASE^{plus} version 2.0 (Biogazelle, Ghent, Belgium).

Western blot analysis

Omental, mesenteric, retroperitoneal, and tail head AT and liver samples (d 105 p.p.) from a control cow in Trial 2 were exemplarily used for characterizing the SAA protein in these tissues. Tissues were homogenized in HEPES buffer (10 mM pH 7.4) using a Precellys® system (Peqlab, Erlangen, Germany). The total protein content was quantified by the bicinchoninic acid method (BCA, Sigma-Aldrich) and equal amounts of protein (15 μg) were loaded in 16% tris-tricine-SDS gels. After electrophoresis, the gels were blotted onto polyvinyl difluoride membranes (GE Healthcare Bio-Science GmbH, Munich, Germany). The membranes were immunolabelled for the presence of SAA using a rabbit anti-bovine SAA antibody (10.1 mg/mL) diluted 1:4000 and the antibody-antigen complexes were tagged with horseradish peroxidase coupled to a secondary goat anti-rabbit IgG antibody (SouthernBiotech, Birmingham, AL). Detection was carried out by enhanced chemiluminescence (GE Healthcare UK limited, Buckinghampshire, UK). To confirm that an equal amount of protein was loaded on each lane, membranes were stripped and immuno-labelled with a mouse anti-human ß-actin antibody (0.5 mg/mL), 1:2000 dilution (BioVision, Mountain view, CA) and horseradish peroxidase -labelled goat anti-mouse IgG (SouthernBiotech).

Statistical analyses

All statistical analyses were performed using SPSS (version 20, SPSS Inc., Chicago, IL). For Trial 1, the mixed model procedure was used. Treatments (Control and CLA) were considered as fixed factor, and sampling dates (time) as a repeated effect; the respective interaction was also included into the model. The covariance structure component symmetry and Bonferroni correction were used. For Trial 2, all data were tested for homogeneity of variances ($P < 0.1$). Homogeneity of variances was significantly different. Thus, non-parametric tests (Kruskal-Wallis or Mann-Whitney) were used. A *P*

value < 0.05 was considered significant.

RESULTS

Trial 1. Liver *SAA3* mRNA abundance was maximal at the day of parturition (8 fold higher in comparison to the mean of the other d antepartum and p.p. but remained fairly stable throughout all the other days (Figure 1). In s.c. AT from tail head, the day of parturition was significantly different in comparison to 196 and 252 d p.p. (7.7 fold higher *SAA3* mRNA abundance). Supplementation with CLA in comparison to the control group yielded no significant effects on *SAA3* mRNA abundance in liver and s.c. AT. To be able to compare *SAA3* mRNA in s.c. AT and liver tissue a similar set of reference genes was needed. Therefore, geometric mean of *POLR2A* and *EIF3K* was used. Taking into account all data across times and treatment, the *SAA3* mRNA abundance was slightly higher (1.25 fold) in s.c. tail head AT than in liver.

Trial 2. Decreasing mRNA abundance of *SAA3* from d 1 to d 42 and 105 was observed in all fat tissues except mesenteric AT in which *SAA3* was constantly expressed (Figure 2). The decrease of *SAA3* mRNA abundance in liver and muscle tissues was significant between d 1 and d 42 and in mammary gland was similar to the AT depots (Figure 3). In pancreatic tissue, the presence of *SAA3* mRNA was confirmed (data not shown), but due to the low quality of the RNA extracted no statistics were done for this tissue. The decline in *SAA3* mRNA abundance from d 1 to d 42 and 105 was different between tissues: s.c. AT from tail head, retroperitoneal, s.c. AT from sternum, s.c. AT from withers, omental, and mesenteric AT had 44.1 > 31.5 > 21.8 > 7.67 > 5.9 > 1.8 fold higher *SAA3* mRNA abundance at d 1 in comparison to the mean of d 42 and 105, respectively. In case of mammary gland, muscle, and liver tissues, the reduction was 34.4 > 7.5 > 1.0 fold, respectively. Supplementation with CLA caused a reduction in the abundance of *SAA3* mRNA in comparison to control group i.e. in s.c. withers AT at d 42 p.p. (5.8 fold decrease), in s.c. AT from sternum at d 105 p.p. (3.33 fold decrease), and in muscle tissue at d 105 p.p. (7.98 fold decrease).

We pooled the data from different days (1, 42, and 105 p.p.) and treatments per tissue to be able to compare the *SAA3* mRNA abundance between different tissues i.e. mammary gland, liver and the 6 different AT using the adequate panel of reference genes (Figure 4). The *SAA3* mRNA had its highest expression in mammary gland i.e. 20 fold higher expression in comparison to the mean of AT and 33 fold higher than liver tissue. The second highest mRNA expression was in omental AT that was 2 to 26 fold greater than in

the other 5 fat depots (Figure 4). Depending on the fat depot, the SAA3 mRNA abundance in AT was either greater or smaller than in liver: the values in two s.c. depots (from tail head and from sternum) and in omental fat exceeded the ones from liver by a factor of 2.4, 1.4, and 5.3, respectively. The values in the remaining depots amounted to 20% (s.c. from withers and retroperitoneal AT) and 40% (mesenterial AT) of the ones in liver.

Culture and differentiation of bovine preadipocytes.

The *SAA3* mRNA expression in an adipose cell culture system was low in non-differentiated adipocytes (d 0) compared to other stages of differentiation. At d 2 of differentiation, *SAA3* mRNA increased by a factor of 91 in comparison to d 0. Thereafter (at d 6, 8, 10, 12, and 13), *SAA3* mRNA abundance decreased to values that were still 10, 3.9, 4, 1.6, and 1.3 fold higher than on d 0 of differentiation (Figure 5).

Detection of the SAA protein in adipose tissues

Expression of SAA protein was confirmed by Western blot analysis in different bovine fat depots (Figure 6). Immunoreactive bands were mainly detected as a 14 kDa monomer. In addition, bands of about 26-28 kDa and 52-56 kDa likely to be the SAA dimer and tetramer were observed. Other faint bands of higher molecular weight probably result from the association of SAA with other proteins e.g. albumin.

DISCUSSION

Expression of *SAA3* mRNA in adipose tissue, mammary gland and liver

When ranking the different tissues investigated herein according to their *SAA3* mRNA abundance, mammary gland had the highest values and was followed by some AT depots exceeding the abundance in liver, whereas other AT depots ranked below liver. This indicates that SAA3 might be important for AT functions. However, secretion of SAA3 from AT yet needs to be clarified. There is no data available showing contribution of mammary *SAA3* to blood; it is mainly secreted to colostrum and milk (Molenaar et al., 2009). Secretion of SAA3 into the medium of a murine adipocyte culture was already shown, but a significant contribution to circulating SAA from AT was considered improbable (Chiba et al., 2009). In murine AT, SAA3 was identified as the major SAA isoform (Sommer et al., 2008). However, there is no selective antibody for bovine SAA3 available; using the anti-bovine SAA antibody, a SAA monomer of comparable size as shown by Dilda et al. (2012) was detectable in different AT at the protein level. Different bands in addition to the monomer are probably related to multimerization and association

with other proteins. Indeed, SAA may form polymers during storage and freezing/thawing of samples (Molenaar et al., 2009). Other factors that might affect structure and stability of SAA are different metal ions and their concentration at least in *in vitro* conditions (Wang and Colón, 2007).

The abundance of *SAA3* mRNA varied considerably between the different bovine AT investigated herein, but explanations for the underlying reasons and functions are lacking. There was no general difference between s.c. and v.c. fat depots and neither the portion of the fat depots nor their readiness for mobilization as concluded from body weight composition at the different sampling days (von Soosten et al., 2011) seem to be related with *SAA3* mRNA abundance.

Expression of *SAA3* mRNA during differentiation of primary bovine preadipocytes

In both undifferentiated and differentiating preadipocytes from s.c. AT of dairy cows, *SAA3* mRNA was expressed at low abundance, whereas a peak value was observed at the onset of differentiation at d 2. We speculate that high expression of *SAA3* in AT, but not in differentiated preadipocytes, might be related to the maturity of the preadipocytes and the degree of fat accumulation. However, our cell culture data support that *SAA3* can indeed be classified as an adipokine originating from adipocytes.

Dexamethasone, one of the components of the differentiation medium, is known to stimulate *SAA3* mRNA (Fasshauer et al., 2004). The increase in *SAA3* mRNA abundance in differentiating bovine preadipocytes at d 2 supports that *SAA3* is positively regulated by glucocorticoids. Thus it might be similarly linked to insulin sensitivity in bovine AT as demonstrated in murine adipocytes by Fasshauer et al. (2004).

To date the biological significance of SAA expression in AT is largely unknown and remains speculative: SAA is an apolipoprotein and is thus involved in binding, transport and targeting of NEFA mobilized during the peripartum-related lipolysis. Besides, SAA may exert direct metabolic effects on human adipocytes by decreasing lipogenesis through an extracellular signal-regulated kinase dependent pathway. In particular, SAA down regulated peroxisome proliferator activated receptor gamma, CCAAT/enhancer binding protein alpha and sterol regulatory element binding protein-1c, which in turn are related to adipocyte differentiation and lipid synthesis (Faty et al., 2012).

Association of *SAA3* mRNA with the inflammatory reaction in dairy cows

In both trials, the maximum expression of AT *SAA3* mRNA was observed in all analyzed tissues at the day of parturition. For liver, we confirmed the recent observation of highest *SAA3* mRNA abundance around parturition in cows (Graugnard et al., 2013). Delivery

related stress, tissue lesions, and inflammatory reactions of the genital tract have been suggested as possible explanations for the acute phase reaction commonly observed at that time (Murata, 2007; Humblet et al., 2006). The involvement of AT in this reaction might thus be due to a systemic stimulation of acute phase protein that occurs in all tissues. Albeit the contribution of SAA3 from AT to the circulating SAA concentrations seems less probable (Chiba et al., 2009), increased *SAA3* expression in AT might have a local importance: inflammatory reactions with increased *SAA3* in AT have been demonstrated to be related to reduced insulin sensitivity in obese mice (Lin et al., 2001, Reigstad et al., 2009). As mentioned previously, reduced peripheral insulin sensitivity is a common feature in early lactating dairy cows and might be at least partly attributable to the locally elevated concentrations of SAA3.

Supplementations with CLA are discussed to be related to inflammatory response and insulin sensitivity (Reynolds and Roche, 2010). The sparse effects of CLA treatment on *SAA3* mRNA abundance observed herein in some fat depots and in muscle were inconsistent with regard to time, tissue and direction of change. Nevertheless, the differences established herein indicate that there might be local effects of CLA in these tissues. Both *SAA3* mRNA and protein are induced during adipocyte hypertrophy and development of obesity in mice (Han et al., 2007; Chiba et al., 2009; Scheja et al., 2008). In view of the effects of CLA reported in rodents and humans, i.e. reducing body fat and fat cell size, a CLA-induced down-regulation of *SAA3* in AT was anticipated. However, from all fat depots investigated herein, the s.c. fat had the lowest share of these depots and the CLA treatment largely yielded no effects on fat depot masses (von Soosten et al., 2011). We observed reduced fat cell sizes in both s.c. and v.c. depots from the CLA treated primiparous cows investigated herein as compared to the controls (Akter et al., 2011). However, this CLA related decrease of cell size coincided with decreased *SAA3* mRNA abundance solely in one of the fat depots investigated, and thus provides only little support for *SAA3* being involved in adipose cellularity. As to whether the CLA effect will remain on the level of the SAA protein remains to be clarified since the expression of SAA protein in different AT of dairy cows was demonstrated in a qualitative but not a quantitative manner.

For cattle, the presence of *SAA3* mRNA shown herein in pancreatic tissues of dairy cows corroborates earlier studies (Lecchi et al., 2012), however, the functional role of SAA3 in pancreas remains to be investigated.

CONCLUSIONS

Herein, we defined SAA3 as a new bovine adipokine. Longitudinal characterization of the *SAA3* mRNA in AT but also in non-fat tissues during late pregnancy and the subsequent lactation yielded a peripartal peak. The sparse effects observed for the CLA treatment on *SAA3* mRNA abundance in s.c. AT and in muscle tissue do not consistently support that local anti-inflammatory effects of CLA might occur, however, this option can also not be entirely ruled out. To clarify potential effects of CLA in this respect, isomer specific treatments would be more meaningful; however, our results for the CLA isomer mixture from this study and from a previous one about another acute phase protein i.e. haptoglobin (Saremi et al., 2012) imply that this form of CLA supplementation that is available in the field, leaves the major bovine acute phase proteins largely unaffected.

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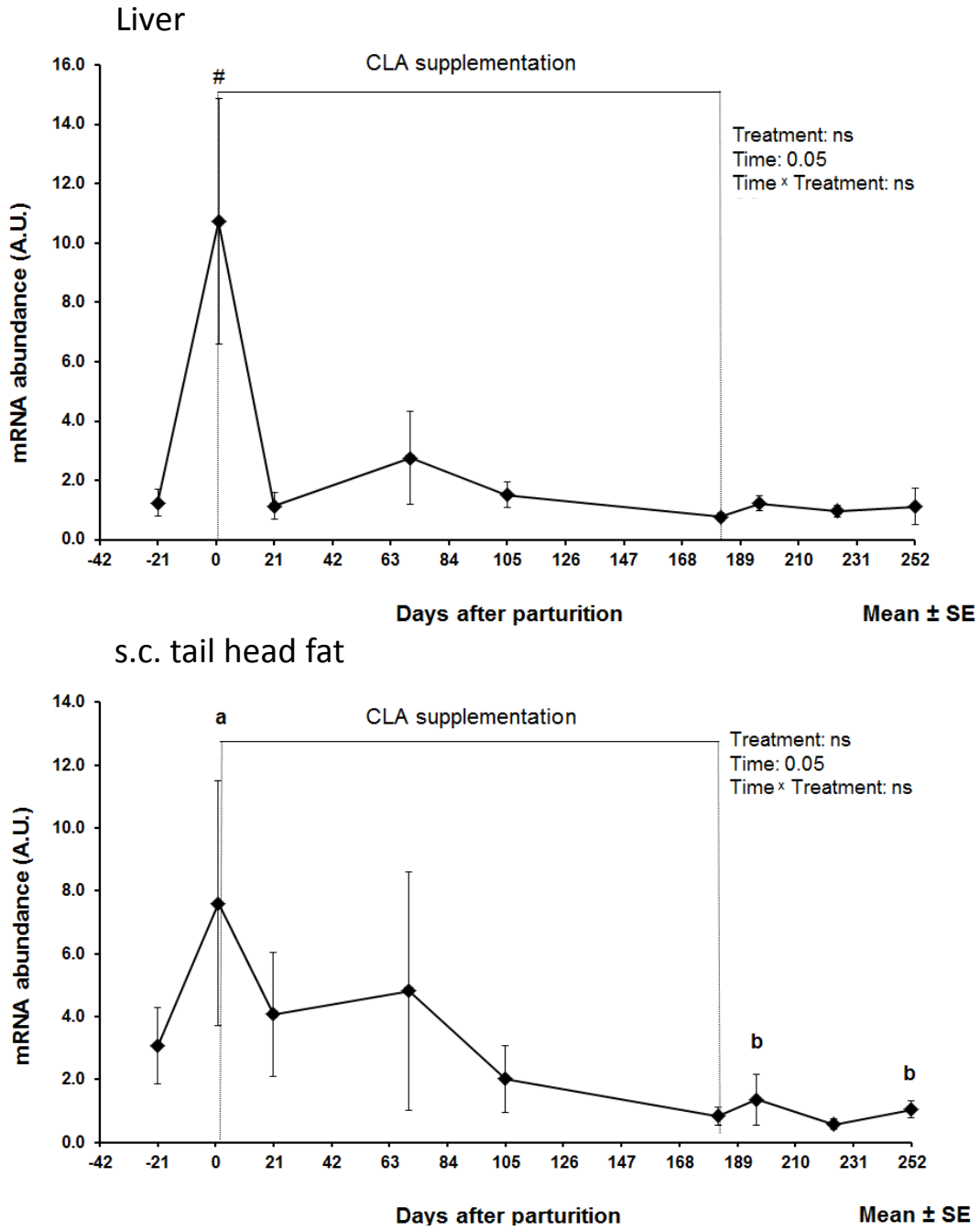

Figure 1. Serum amyloid A3 (*SAA3*) mRNA abundance (means ± SEM) in liver and in subcutaneous tail head fat of pluriparous cows. Based on the lack of significant treatment effects, data were pooled from cows receiving conjugated linoleic acids (CLA, Lutrell® Pure, BASF SE, Ludwigshafen, Germany; n = 11) at 100 g/d and from cows receiving a control fat supplement (Silafat®, BASF SE; n = 10) from d 1 until d 182 postpartum in Trial 1. Samples from d 1, 70, 182, and 224 were limited to the control cows. In liver, # defines a significant difference in comparison to all the other sampling dates ($P < 0.05$). Different letters indicate significant differences between d relative to parturition ($P < 0.05$). Data are normalized based on the geometric mean of 3 most stable reference genes: lipoprotein receptor-related protein 10 (*LRP10*), RNA Polymerase II (*POLR2A*) and eukariotic translation initiation factor 3 (*EIF3K*) in liver and *LRP10*, glyceraldehyde-phosphate-dehydrogenase (*GAPDH*), and *POLR2A* in s.c adipose tissue. A.U.: arbitrary units.

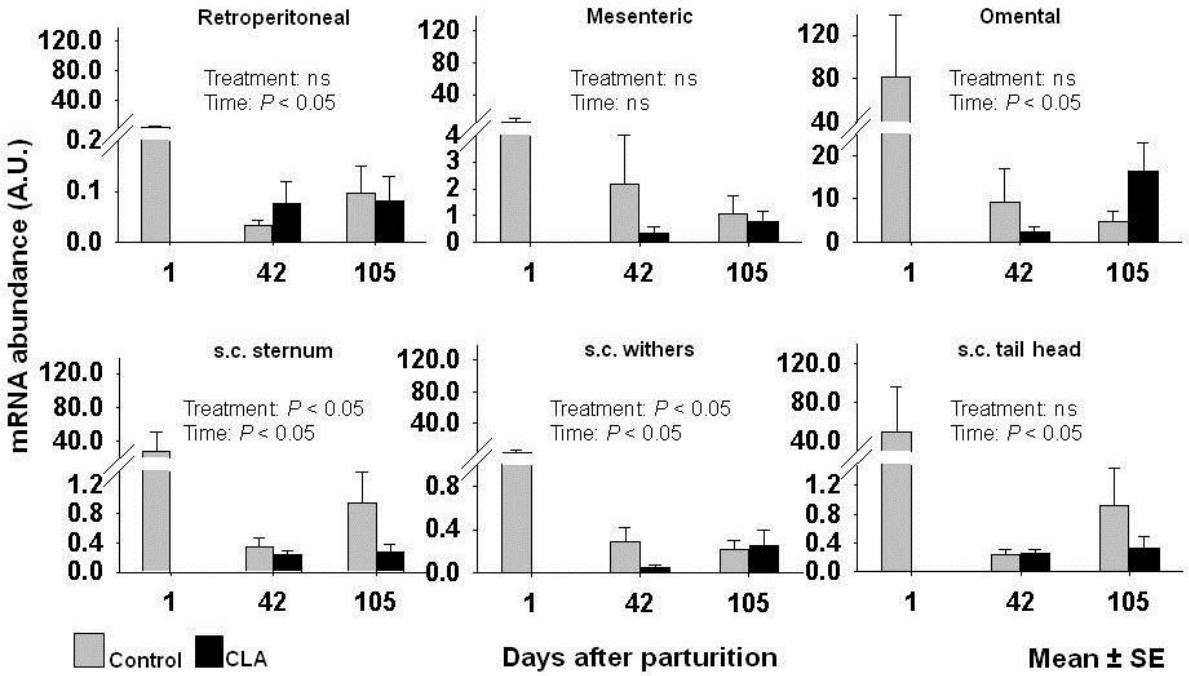

Figure 2. Serum amyloid A3 (*SAA3*) mRNA abundance (means ± SEM) in three visceral (upper panels) and three subcutaneous fat depots (lower panels) in primiparous cows supplemented with 100 g/d conjugated linoleic acids (CLA, Lutrell® Pure, BASF SE, , Ludwigshafen, Germany) or a control fat supplement (Silafat®, BASF SE) from d 1 until d 105 postpartum. Within the control group, *SAA3* mRNA abundance was higher ($P < 0.05$) on d 1 than on d 42 and d 105 in retroperitoneal and omental fat. In subcutaneous fat (s.c.) from sternum, withers, and tail head the difference was limited to d 1 vs. d 42 and in omental fat there was an additional decrease from d 42 to 105. The effects of CLA treatment were limited to s.c. fat in which reduced *SAA3* mRNA abundance was observed at d 42 and 105 in s.c. fat from withers and sternum, respectively. ns: not significant. Data are normalized based on the geometric mean of the most stable reference genes: Eukariotic translation initiation factor 3 (*EIF3K*), Lipoprotein receptor-related protein 10 (*LRP10*), RNA polymerase II (*POLR2A*), Emerin (*EMD*), Marvel domain containing 1 (*MARVELD1*), and Hippocalcin-like 1 (*HPCAL1*) for each s.c. fat and mesenteric fat depots and *EIF3K*, *LRP10*, *POLR2A*, *EMD*, and *MARVELD1* for omental and retroperitoneal fat depots. A.U.: arbitrary units.

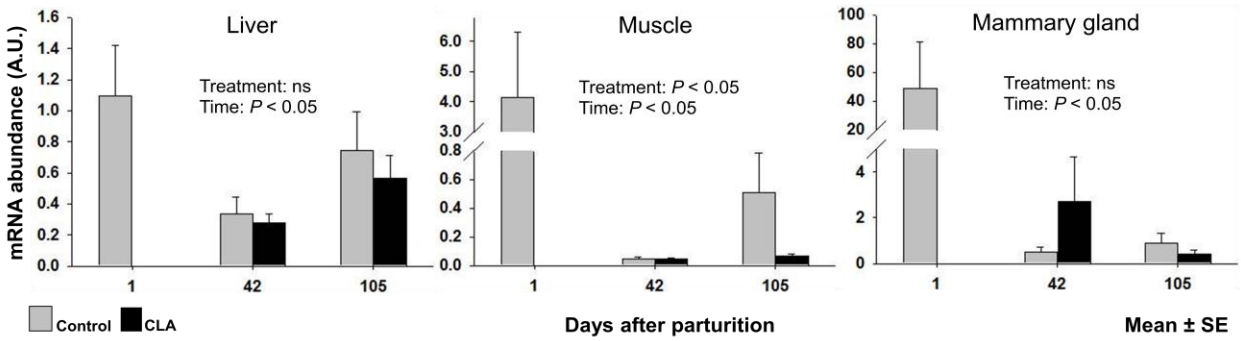

Figure 3. Serum amyloid A3 (*SAA3*) mRNA abundance (means ± SEM) in liver, *M. semitendinosus*, and mammary gland of primiparous cows receiving conjugated linoleic acids (CLA, Lutrell® Pure, BASF SE, Ludwigshafen, Germany) at 100 g/d or a control fat supplement (Silafat®, BASF SE) from d 1 until d 105 postpartum. Significant differences between sampling dates within tissue of the control group were between d 1 and d 42 in liver, muscle, and mammary gland ($P < 0.05$). At d 105, *SAA3* mRNA abundance remained low in mammary gland, but increased to the values of d 1 in liver and muscle tissue. Effects of CLA were limited to d 105 postpartum in muscle tissue. Data are normalized based on the geometric mean of the most stable reference genes: Hippocalcin-like 1 (*HPCAL1*), Lipoprotein receptor-related protein 10 (*LRP10*), RNA polymerase II (*POLR2A*), Eukariotic translation initiation factor 3 (*EIF3K*), Glyceraldehyde-phosphate-dehydrogenase (*GAPDH*) for liver, *LRP10*, Emerin (*EMD*), *POLR2A*, *EIF3K* for muscle, and Marvel domain containing 1 (*MARVELD1*), *EMD*, *LRP10*, *EIF3K*, *POLR2A*, *HPCAL1* for mammary gland tissue. A.U.: arbitrary units.

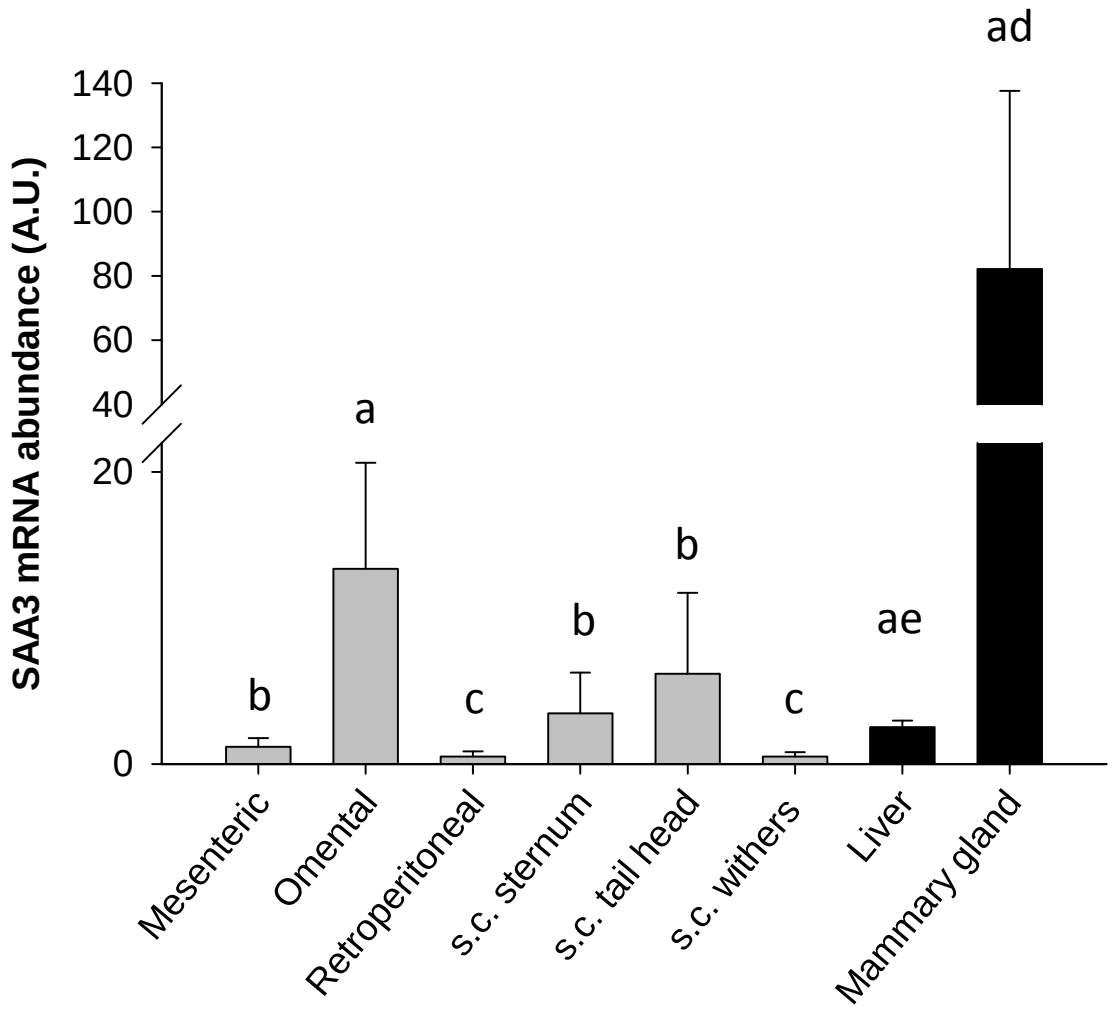

Figure 4. Serum amyloid A3 (*SAA3*) mRNA abundance (means ± SEM) in different fat and non-fat tissues of primiparous cows across all sampling dates and treatments. Different letters specify the differences between the individual fat depots ($P < 0.05$). Data are normalized based on the geometric mean of 4 most stable reference genes: Eukariotic translation initiation factor 3 (*EIF3K*), Lipoprotein receptor-related protein 10 (*LRP10*), RNA polymerase II (*POLR2A*), and Hippocalcin-like 1 (*HPCAL1*). A.U.: arbitrary units.

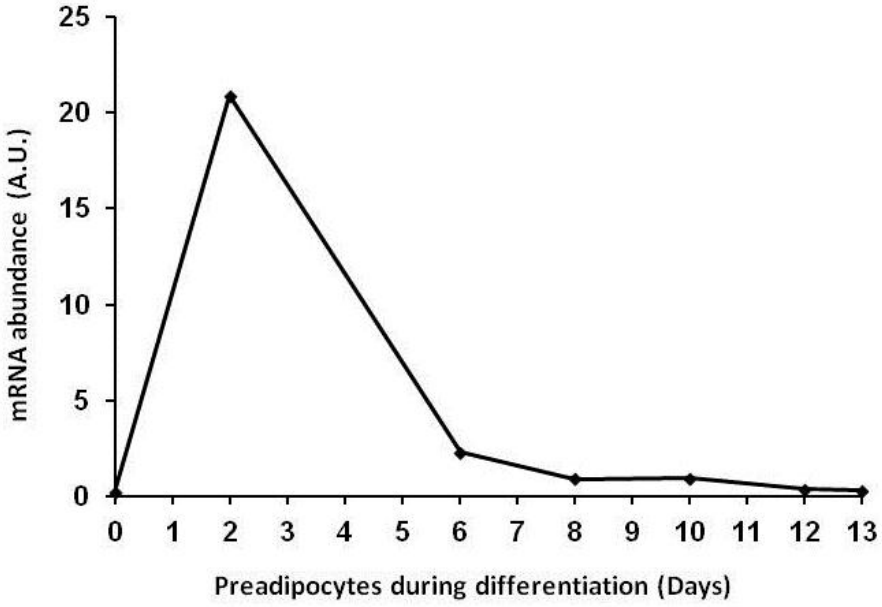

Figure 5. Timely changes of Serum amyloid A3 (*SAA3*) mRNA abundance in bovine preadipocytes differentiating for 13 d. Primary cells were obtained from 3 individuals and were pooled; samples from the given days of differentiation were assayed in triplicates. The mRNA quantities were normalized to the geometric mean of two reference genes: Glyceraldehyde-phosphate-dehydrogenase (*GADPH*) and Lipoprotein receptor-related protein 10 (*LRP10*). A.U.: arbitrary units.

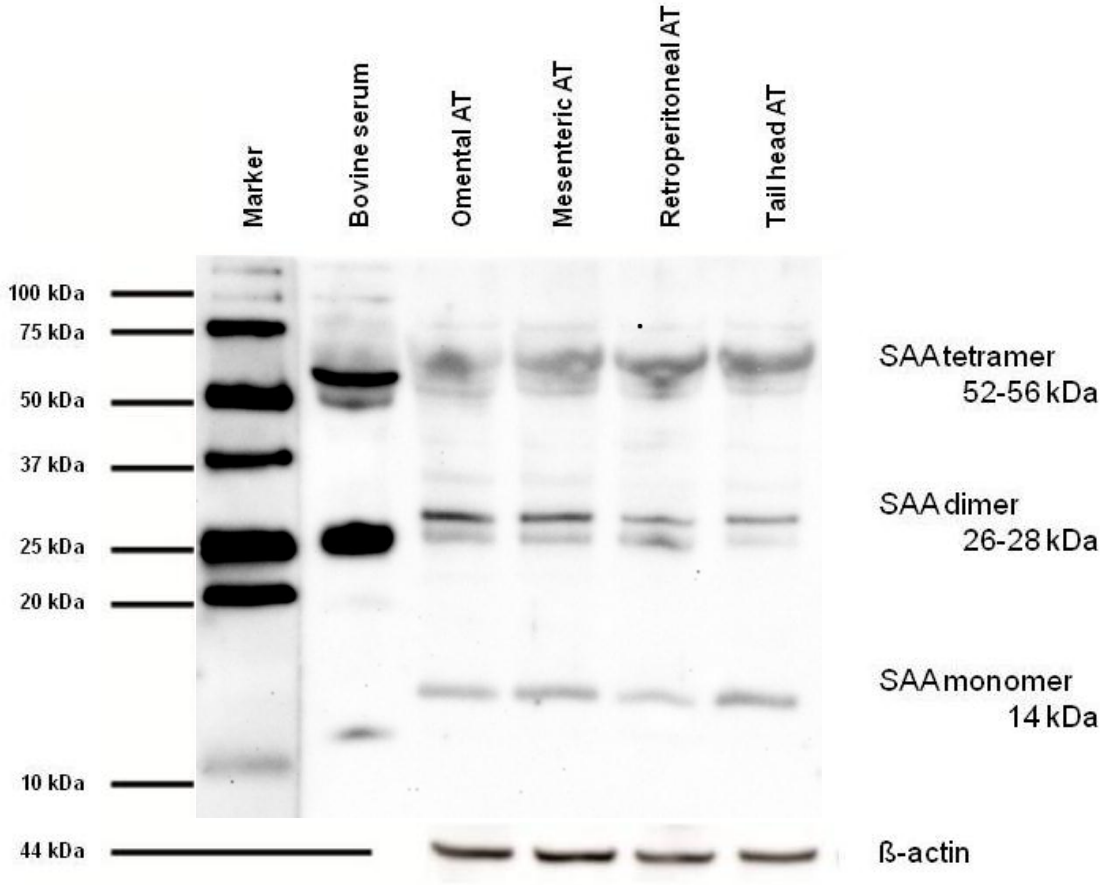

Figure 6. Tris-tricine-SDS gel and Western blot analysis of adipose tissue homogenates from different fat depots. Equal amounts of total protein (15 µg) of each tissue homogenate were loaded. SAA was detected using a rabbit anti-bovine SAA antibody. Bovine serum was used as a positive control. To confirm that an equal amount of protein was loaded on each lane, membranes were stripped and immuno-labelled with a mouse anti ß-actin antibody. AT: adipose tissue.

6. Manuscript 4 (submitted, under revision)

Longitudinal characterisation of insulin sensitivity related gene expression in dairy cows during the lactation period focusing on adipose tissues

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Abstract

In dairy cows the milk associated output of energy in early lactation exceeds the input via voluntary feed intake. To spare glucose for mammary synthesis of lactose, insulin sensitivity (IS) in peripheral tissues is reduced and the mobilization of body reserves, mainly body fat, is stimulated. For these processes a link between IS and the endocrine functions of adipose tissue (AT) is likely and we thus aimed to characterize the time course of mRNAs from bovine AT derived proteins and receptors related to IS in metabolic active tissues as well as systemic IS throughout lactation. Conjugated linoleic acids (CLA) reduce milk fat thus decreasing the milk drain of energy and potentially dampening lipolysis, but may also affect IS. Biopsies (subcutaneous (s.c.) AT and liver) of pluriparous cows receiving a control fat or a CLA supplement (each at 100 g/day from 1 to 182 days in milk) covering 3 weeks antepartum until 36 weeks postpartum as well as samples from liver, mammary gland, skeletal muscle and 3 visceral and 3 s.c. AT were obtained from primiparous cows treated analogously and slaughtered on days in milk 1, 42 or 105. Tissue samples were assayed for mRNA abundance of adiponectin, its receptors, leptin, leptin receptor, PPARγ and PPARγ2, IL-6, and TNF-α.

For most of the targets, changes throughout lactation, albeit heterogeneous, were observed. The gene expression was different between AT depots. In s.c. AT of pluriparous cows most mRNAs decreased peripartum, except TNF-α. In primiparous cows most changes were related to adiponectin receptor 1, with lowest expression on 1 day in milk. In primiparous cows, CLA effects were largely limited to decreased PPARγ2 mRNA abundance in the mammary gland. In pluriparous cows, systemic IS was reduced by CLA treatment but without consistent changes in tissue mRNA abundance of the target genes.

Introduction

Late pregnancy and lactogenesis are linked to comprehensive endocrine and metabolic changes of the female. Homeorhetic adaptations are necessary to accomplish the nutrient drain towards the mammary gland. They are associated with decreasing peripheral insulin sensitivity (IS) in peripheral tissues other than the mammary gland in dairy cattle [1] just as in other mammalian species like humans [2]. Nevertheless, genetic selection for milk synthesis in dairy cattle results in a particular distinctive negative energy balance (NEB) which is often associated with metabolic diseases like fatty liver [3].

Messenger molecules derived from adipose tissue (AT), i.e. adipokines, are important

metabolic regulators and modulate IS [4].This is well known for monogastric species in contrast to ruminants. The adipokines are related to energy metabolism (e.g. adiponectin (ADIPOQ) and leptin (LEP)) as well as inflammation (e.g. tumor-necrosis factor-α (TNF-α) and interleukin-6 (IL-6) [5]. The adipokine ADIPOQ reduces gluconeogenesis, leading to decreased hepatic glucose release; glucose uptake as well as beta-oxidation by liver and muscle is also increased [6,7]. Activation of inflammatory pathways in AT and release of inflammatory cytokines from AT can affect IS directly at the level of insulin sensing, e.g. reduced phosphorylation of insulin receptor and insulin receptor substrate-1 lead to decreased glucose uptake as shown for TNF-α [8] and IL-6 [9].

The transcription factor peroxisome proliferator-activated receptor gamma (PPARγ) is a central regulator of genes related to insulin sensitivity, including ADIPOQ, IL-6 and TNF-α [10,11]. In dairy cattle it was shown that gene expression of PPARγ is linked to lipogenic gene expression in mammary gland [12] and the PPARγ agonist 2,4-thiazolidinedione increases plasma glucose [13]. In cattle, two isoforms of PPARγ (PPARγ1 and PPARγ2) sharing 90% identity are described [14]. In general, the PPARγ2 isoform is highly expressed in AT; it plays an important role in adipocyte differentiation and regulates IS [15].

As recently reviewed, AT from different areas of the body display distinct structural and functional properties including adipokine secretion. In humans and rodents, visceral (v.c.) AT feature higher lipogenic and lipolytic activities and produce more pro-inflammatory cytokines, while subcutaneous (s.c.) AT are the main source of LEP and ADIPOQ, and thus have disparate roles, amongst others in IS [16]. So far, studies in dairy cows were mostly limited to s.c. fat which can readily be biopsied.

Linoleic acid and their derivatives like conjugated linoleic acids (CLA) are ligands for PPARs [17,18]. In 3T3L1 adipocytes *trans*-10, *cis*-12 CLA decreases ADIPOQ in both PPARγ dependent and independent mechanisms *in vitro* [19]. CLA is also a ligand for free fatty acid receptor 1 and activation of the receptor increases glucose stimulated insulin secretion in mice [20]. From rodent studies using high doses (up to 1% of the diet) of *trans*-10, *cis*-12 CLA, dramatic loss of AT, inflammation, insulin resistance and hepatic steatosis are reported [21]; however, when using lower doses, the effects were largely limited to reduced AT lipogenesis. In dairy cattle, CLA may act energy-sparing as the *trans*-10, *cis*-12 CLA isomer is related to milk fat depression whereas whole body seems largely unaffected during both short and long-term treatments with this CLA isomer [22]. Milk fat depression may improve NEB in dairy cows but the effects are not

conclusive yet as discussed by Hötger et al. [23]. This group has shown that the main isomers (*trans*-10, *cis*-12 CLA, *cis*-9, *trans*-11 CLA) decreases endogenous glucose production and milk fat synthesis but increases plasma glucose, whereby it had no effect on insulin secretion in cows during their second lactation.

Knowledge on IS related gene expression in relevant tissues involved in energy metabolism in dairy cattle may help to reduce the incidence for metabolic diseases in future. Therefore we aimed to characterise the time course of the expression of IS related genes during lactation in different AT of v.c. and s.c. localisations in addition to muscle, liver and mammary gland. Based on the potential of CLA on energy-sparing in addition to affect glucose metabolism and possibly IS, we supplemented primiparous and pluriparous Holstein cows' diet with a 1:1 mixture of *trans*-10, *cis*-12 CLA and *cis*-9, *trans*-11 CLA which is registered as feed supplement in the EU. Subcutaneous AT can readily be biopsied, and therefore we were particularly aiming to compare the mRNA expression between different s.c. and v.c. AT depots available through timed slaughter of primiparous cows. The target genes we selected herein are related to IS, and we therefore aimed to test not only tissue mRNA expression but also to estimate systemic IS using the "Revised Quantitative Insulin Sensitivity Check Index" (RQUICKI) that was suggested for use in dairy cows [24].

Materials and Methods

The experiments and treatments of the animals were approved by the competent authority, the lower saxony state office for consumer protection and food safety (LAVES, file no. 33.11.42502-04-071/07, Oldenburg, Germany). The regulations of the German Animal Welfare Act (TierSchG) in its respective current edition were met. All animals were housed in free stall barns at the experimental station of the Friedrich Loeffler Institute, Federal Research Institute for Animal Health, Braunschweig, Germany.

Animals, diets and treatments

Trial 1. German Holstein pluriparous (n = 21) and primiparous (n = 10) cows were fed a partial mixed ration consisting of 38% grass silage, 25% corn silage and 37% concentrate on a dry matter basis for ad libitum intake over the entire experimental period. Diets were formulated according to the recommendations of the German Society of Nutrition Physiology [25]. More details about the experiment and the outcomes in terms of performance, metabolite concentrations and milk FFA profile from this experiment are

described by [26]. On DIM 1 the animals were allocated to 2 groups: a CLA group (11 pluriparous and 5 primiparous cows) and a control group (10 pluriparous and 5 primiparous cows). During the supplementation period (from 1 to 182 DIM), the animals received 4 kg of additional concentrate containing the fat supplements. The animals of the CLA group received 100 g/d encapsulated CLA (Lutrell Pure, BASF, Ludwigshafen, Germany). The control group received 100 g/d of a control fat supplement (Silafat, BASF) in which CLA were substituted by stearic acid. In the CLA group, the animals consumed 7.6 g/day each of the *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA isomers. Liver and s.c. AT from tail head were biopsied on d 21 before calving and on DIM 1, 21, 70, 105 182 196, 224, and 252. The biopsy procedures were previously described in detail by Saremi et al. [27]. For gene expression analyses, samples were restricted to the pluriparous cows of the control group (all sampling dates) and of the CLA group (d -21, 21, 105, 196, and 252) as the focus of the study was related to changes throughout lactation and the capacity of handling the samples was limited. Jugular blood samples were collected on d - 21, - 14, - 7, 1, 7, 14, 21, 35, 49, 70, 105, 140, 182, 189, 196, 210, 224, 238, and 252 relative to calving.

Trial 2. German Holstein primiparous cows (n = 25, average age at calving was 23 months) were fed a partial mixed ration (ad libitum) according to the recommendations of the German Society of Nutrition Physiology [25]. The ration consisted of 63% silage (60% corn silage, 40% grass silage) and 37% concentrate on a dry matter basis. Five cows were slaughtered on 1 DIM and the remaining animals (n = 20) were randomly allocated to either receive 100 g/d of the control fat supplement (Silafat, BASF) or of CLA (Lutrell Pure, BASF) starting from 1 DIM. All experimental details and main outcomes in terms of performance, adipose depot and organ weights, body composition, body fat mobilization, protein accretion, and energy utilization were reported by von Soosten et al. [28,29]. Five cows per group were slaughtered at 42 and 105 DIM. Samples from 3 v.c. AT (omental, mesenteric, and retroperitoneal), 3 (tail head, withers, and sternum), liver, *M. semitendinosus*, pancreas, and mammary gland were taken immediately after slaughter. Blood samples were collected from *V. jugularis* on d - 21, - 14, - 7, - 3, 1 (n = 25), 7, 14, 28, 42 (n = 20), and 105 (n = 10) relative to parturition.

The tissue samples from both trials were snap-frozen in liquid nitrogen and stored at - 80 °C for RNA extraction and analysis. The blood samples were collected in different tubes to obtain sodium fluoride/ EDTA plasma, heparin plasma or serum. Plasma samples were stored at - 20 °C and sera at - 80 °C until analysed.

Relative quantification by real-time PCR

Preparation of samples including RNA extraction and cDNA synthesis together with real-time PCR and reference genes characteristics, selection, and measurements are described in detail by Saremi et al. [27,30]. Relative quantification of the target genes was performed as summarized in table 1. Amplicon standard curve was used except for *PPARγ2* in Trial 1 and 2 and *LEPRB* and *TNF-α* in Trial 1 where cDNA standards curve was used. The standard curve was used to correct data based on PCR efficiency per run. Two µL cDNA (diluted 1 to 4) as template and 5 µL SYBR Green JumpStart Taq Readymix (Sigma-Aldrich, Steinheim, Germany) in a total volume of 10 µL were run in an Mx3000P real-time PCR cycler (Stratagene, Amsterdam, Netherlands and Agilent, Santa Clara, CA).

Reference gene stability and data analysis

In each individual case, comparing treatment effects within a single tissue or comparing different tissues, an adequate panel of reference genes was selected according to Saremi et al. [27,30].

Trial 1. The reference genes used were low density lipoprotein receptor-related protein 10 (LRP10), glyceraldehyde-phosphate-dehydrogenase (GAPDH), and RNA Polymerase II (POLR2A) for s.c. AT and LRP10, POLR2A, and eukariotic translation initiation factor 3, subunit K (EIF3K) for liver tissue according to Saremi et al. [27]. The geometric mean of the reference gene abundances was used for normalization. Data are presented as ratio of the copy numbers of genes of interest and the geometric mean of the corresponding reference genes.

Trial 2. The quantification cycle values were imported to qBASE^{plus} version 2.0 (Biogazelle, Ghent, Belgium) and all subsequent calculations and data quality controls were done based on this software [31]. Reference genes were tested per tissue and the lowest V value obtained for the number of reference genes needed for normalization as described by Saremi et al. [30]. Briefly, for normalization of the efficiency corrected data the reference genes EIF3K, LRP10, POLR2A, Emerin, Marvel domain containing 1 and Hippocalcin-like 1 were selected and used, depending on the tissues being compared [30] were used. The data used for statistics was generated by geNorm^{plus} as a part of qBASE^{plus} version 2.0 (Biogazelle, Ghent, Belgium).

Analysis of NEFA, BHBA, LEP, and insulin in blood samples

Glucose was measured in sodium fluoride/ EDTA plasma, for quantification of insulin, NEFA, and BHBA heparinized plasma was used. The plasma concentrations of glucose, NEFA, and BHBA were determined by an automatic analyser (Cobas Mira Plus System from Roche Diagnostica Ltd, Basel, Switzerland) using commercial test kits (Glucose: Glucose Hexokinase Fluid 5+1, MTI Diagnostics GmbH, Idstein, Germany; NEFA: HR(2) R1+R2 Set, WAKO Chemicals GmbH, Neuss, Germany; BHBA: RANBUT, RB 1008, Randox Laboratories GmbH, Wülfrath, Germany). LEP concentrations were determined in serum by ELISA [32]. The intra and inter assay coefficients of variations were 6.3% and 13.9%, respectively, the limit of detection was 0.3 ng/mL. The concentrations of insulin in plasma were measured using a commercially available double antibody radioimmunoassay (DSL-1600, Diagnostic Systems Laboratories, Inc., TX). The intra and inter assay coefficients of variation were 6.3% and 8.8%, respectively.

Calculations and statistical analyses

The RQUICKI was calculated based on the concentrations of plasma glucose, NEFA, and insulin (Perseghin et al., 2001) as previously used to estimate IS in dairy cows [24] by the following equation:

$$RQUICKI = 1 / [\log(glucose) + \log(insulin) + \log(NEFA)].$$

All statistical analyses were performed using SPSS (version 20, SPSS Inc., Chicago, IL). For Trial 1, the mixed model procedure was used. Treatment (control and CLA) was considered as fixed factor, sampling dates (time) as repeated effect, and the respective interaction was included in the model. For serum LEP, but not for mRNA analyses which were limited to the cows from Trial 1, parity (cows or heifers) was considered as fixed effect together with its interaction with treatment. The covariance structure heterogeneous first-order autoregressive and Bonferroni correction were used for serum data, while component symmetry was used for mRNA data.

For Trial 2 in which the repeated design for tissue samples was not applicable, all data were tested for homogeneity of variances ($P \leq 0.1$). Accordingly the general linear model with the fixed effects of treatment, dates, and the respective interaction, or non parametric tests (Kruskal-Wallis or Mann-Whitney) were applied ($P \leq 0.05$). All data are presented as mean ± SEM.

For both trials $P \leq 0.05$ was considered significant.

Results

Trial 1

During the transition from late pregnancy to early lactation (transition period) in pluriparous cows, in the majority of the cases the mRNA abundance of the target genes was decreased in s.c. AT (Figures 1, 2, and 3). In detail, the mRNA abundance for LEP, LEPR, ADIPOQ, ADIPOR1, ADIPOR2, PPARγ, and PPARγ2 was decreased in s.c. AT. Similarly, the amount of PPARγ and IL-6 mRNA in liver was reduced. In contrast, the quantity of LEPR and LEPRB as well as ADIPOR2 mRNA in liver increased during the transition period. The mRNAs for PPARγ, PPARγ2 and LEPR in s.c. AT were lower throughout the analysed time period up to 252 DIM, compared to a.p.. The abundance of LEPR and LEPRB mRNA in liver was higher up to day 196 DIM compared to a.p., and decreased thereafter to values comparable to the situation a.p., but remained higher at day 252 in case of LEPR. The mRNA quantity for ADIPOR2 in liver was higher at 21 DIM vs. -21 DIM and decreased to the end of the analysed period. No peripartal and lactation related differences were observed for TNF-α in liver and s.c. AT. The pattern of serum LEP followed roughly the mRNA throughout the transition period but these changes were not significant (Figure 2).

Supplementation with CLA only sporadically increased or decreased the expression of few genes at discrete time points in liver and s.c. AT; however, no consistent changes were observed. Treatment with CLA increased the mRNA abundance of LEPRB and TNF-α mRNA abundance in liver, whereas PPARγ mRNA was lower in CLA cows as compared to the controls at defined time points throughout lactation. Lower values than in the control group were also observed at 196 DIM i.e. beyond the supplementation period in case of PPARγ2 in s.c. AT of CLA cows (Figure 3).

Analysing CLA effects on insulin, glucose, and NEFA, and for comparing RQUICKI values in primiparous vs. pluriparous cows, CLA effects were evident only in pluriparous cows. In these animals insulin concentrations were greater and RQUICKI data were lower (8% or 17% during supplementation and depletion period, respectively) compared to the control group (Figure 4).

Trial 2

Comparing time related changes throughout lactation in different adipose depots, liver, muscle, and mammary gland of primiparous cows (Table 2 and 3), the most temporal changes were observed for ADIPOR1. In mammary gland and all fat depots except

omental AT, the receptor mRNA abundance was increased with DIM. In contrast, the amount of this mRNA was decreased in muscle or reduced at d 42 in liver tissue. The mRNA abundance of ADIPOR2 was increased with DIM in retroperitoneal AT and higher at d 105 vs. d 42 in mammary gland. ADIPOQ mRNA abundance increased in two v.c.AT (omental and retroperitoneal) with DIM. LEPRB mRNA abundance was decreased with DIM in all v.c.AT and in the mammary gland. No changes were found for LEP mRNA in primiparous cows throughout lactation. The mRNA abundance for PPARγ and PPARγ2 changed in some tissues but not consistently. For PPARγ an increase over time was detected in omental AT, and for PPARγ2 in retroperitoneal AT. In addition, an increase was observed in liver for both transcripts. The amount of TNF-α mRNA increased in all of the s.c. AT and in liver; in mammary gland and in retroperitoneal AT, a decrease with DIM was observed. IL-6 mRNA was decreased in s.c. AT from tail head and withers but increased in liver over the time.

Supplementation with CLA only had few and inconsistent effects on gene expression in primiparous cows. The mRNA abundance of TNF-α mRNA was reduced at 42 DIM in mesenteric AT, the ADIPOQ and ADIPOR2 mRNA abundance was declined at 105 DIM in omental and retroperitoneal AT, respectively. The mRNA abundance of PPARγ2 was reduced by CLA only in the mammary gland but at both time points after parturition: DIM 42 and DIM 105.

Differences in gene expression of target genes between adipose tissue depots and in relation to other tissues

The mRNA abundance of the target genes was compared between different AT from Trial 2 irrespective of treatment and DIM (Figure 5). Differences between the different AT depots were seen for all target mRNAs except ADIPOR2. In addition, the mRNA abundance of most target genes was different when comparing only within v.c. depots or within s.c. depots.

By the real-time PCR protocol applied, ADIPOQ mRNA in the mammary gland was detectable only in one third of the samples. The abundance was about 900 times lower than in AT. For ADIPOR1, a 2 fold lower abundance was observed in the liver than in the average of all AT whereas in muscle the values were 3.7 fold higher than in AT. The abundance of ADIPOR2 mRNA in muscle was higher than in liver. In both tissues the abundance was mostly in the same range as in the different AT depots although liver and muscle had lower and higher ADIPOR2 mRNA abundance in comparison to

retroperitoneal and mesenteric AT, respectively. Compared to AT, there was about 100 fold less PPARγ2 mRNA in liver, 79 fold less in muscle and 17.4 times less in mammary gland. PPARγ mRNA abundance in liver was 109 fold lower in comparison to the mean of AT. LEP mRNA abundance was low in muscle and mammary gland in comparison to the mean of fat depots (171 and 353 fold lower, respectively). In liver, 34.4 times more LEPRB mRNA abundance than in AT was observed. About 5 fold more TNF-α mRNA was observed in liver compared to AT, but IL-6 mRNA abundance was 8.3 times lower in liver as compared to AT.

Discussion

The incidence for metabolic diseases (e.g. ketosis and fatty liver) during early lactation in dairy cattle is high. The animals pass a period of enormous lipolysis and substantial insulin resistance [3]. Increasing information about the regulation of IS associated genes may help to optimize management strategies improving the health status of the animals in future. In this study, we focused on the mRNA abundance of different genes related to IS in early lactation of primiparous cows and throughout a whole lactation cycle in pluriparous cows in a descriptive manner. The aim was to broaden the knowledge about the regulation of these genes depending on the lactational stage. Differences in gene expression between different AT depots, even between individual s.c. AT localisations were disclosed. The physiological significance of functional differences between s.c. and v.c. AT localisations and its impact on metabolism by its expression profiles were discussed in a broad manner in monogastrics [33]. In humans, v.c. AT compared to s.c. AT is preferably lost during energy restriction and exercise. Nevertheless, differences regarding to mobilisation were described for s.c. localisations, too [34] but especially visceral AT mass is correlated with IS [16]. However, data on gene expression in AT samples of dairy cattle during lactation are mostly restricted to s.c. AT and commonly to a single localisation, typically tail head in case of repeated biopsy sampling.

Supplementation with CLA (50% of each *cis*-9, *trans*-11 and *trans*-10, *cis*-12 CLA), which is discussed to affect IS in monogastrics [35] and is known to decrease milk fat synthesis in ruminants [22], was used to check their ability to influence IS related genes in dairy cattle. We herein aimed to test whether CLA treatment as management strategy to reduce milk fat might alter the expression of IS related genes in various tissues and also the systemic IS. We analysed different s.c. AT depots as well as different v.c. AT depots in dairy cattle to get a broad overview about the flexibility of AT responses in dairy

cattle.

Gene expression of the ADIPOQ and LEP system during lactation

The adipokines LEP and ADIPOQ increase IS. LEP is positively and ADIPOQ is negatively linked with body fat content [7,36]. LEP mainly exerts its effects through LEPRB [37] and ADIPOQ via ADIPOR1 and ADIPOR2, as observed in humans [6]. The blood concentrations of LEP in dairy cattle are low in early lactation [38]; for ADIPOQ less information is available in cattle but decreased concentrations were recently documented using semiquantitative Western blots [39,40] and a new bovine specific ELISA [40].

Recently, Koltes and Spurlock [41] observed a decrease of the ADIPOQ mRNA abundance in s.c. AT from tail head throughout the transition period. This result is comparable to our observation in Trial 1 about ADIPOQ mRNA expression in pluriparous cows and relevant for further genes of the ADIPOQ and LEP system in s.c. AT but also for others like PPARγ and PPARγ2 during the peripartal period. For the LEPR mRNA in s.c. AT, our present results are not in line with data published by our group and others [42,43]. In these two studies, an increase in LEPRB and LEPR mRNA, respectively was observed and explained by Thorn et al. [43] at least partly by the low insulin concentrations during early lactation. As expected, we also observed low insulin concentrations after calving. Other factors might contribute to the regulation of LEPR, which have to be explored in future.

In monogastrics, ADIPOQ and its receptors are inversely related to body fat mass; both receptors but also serum ADIPOQ are down-regulated by insulin in lean men [6,44]. Therefore, the reduction in mRNA abundance of the ADIPOQ system we observed during the transition period in s.c. AT of pluriparous cows in Trial 1 indicates an uncoupling between energy balance and the ADIPOQ system as they were not regulated contrary to the energy status and insulin values as shown in monogastrics [6,44]. In addition, ADIPOQ is associated with lipogenesis and differentiation during adipogenesis at least in murine 3T3-L1 adipocytes [45]. Therefore, the decreased ADIPOQ mRNA abundance in AT during early lactation might be related to reduced lipogenesis and adipogenesis during NEB in discrete AT depots. The regulation of both receptors in liver differs from s.c. AT as the ADIPOR1 mRNA abundance was stable and the one for ADIPOR2 increased in liver during the transition period. Based on ADIPOQ signal transduction in liver as discovered in a mouse model with targeted disruption of the

ADIPORs [46], reduced ADIPOQ serum concentrations during the transition period may enhance gluconeogenesis. The mRNA of ADIPOR2 increased after parturition. Comparable observations are reported by Loor et al. [47]. In their study, the expression of the receptor mRNA was further increased by feed restriction a.p. In addition, growth hormone (GH) might be related to this observation: GH knowingly peaks around parturition in dairy cattle [48] and ADIPOR2 but not ADIPOR1 was shown to be upregulated by GH in liver of mice [49]. Signalling by ADIPOR2 is associated with the reduction of oxidative stress in liver [46], and oxidative stress is increased by NEFA as shown in human hepatocytes [50]. During NEB in early lactation, NEFA concentrations increase [3]. Based on the fact that targeted receptor disruption in liver of mice increases oxidative stress parameters [46], upregulation of the receptor may help to mitigate oxidative stress in liver of dairy cows.

In Trial 2, the most changes throughout lactation occurred for ADIPOR1 mRNA in several adipose depots, in particular in the s.c. AT depots of these primiparous cows in which the abundance increased with DIM. As mentioned earlier, this might be also related to the effects of ADIPOQ on adipogenesis and lipogenesis [45] which are down-regulated during NEB in contrast to lipolysis [51]. As discussed by Dagon et al. [52] and Rossmeisl et al. [53], AMPK is an important mediator of hormonal and nutritional effects on AT and is thus related to the control of body fat mass. Its activation involves the down-regulation of lipogenic and adipogenic genes like PPARγ. Both ADIPORs are linked to AMPK activation but ADIPOR1 to a higher extent than ADIPOR2, which is more associated with PPARα signalling, at least in liver [46]. Therefore, an increase in abundance of ADIPOR1 up to peak lactation might be related to an increase in AMPK activity, controlling lipolysis tightly in retroperitoneal AT and in different s.c. AT of primiparous cows. Based on recent studies, the energy sensor AMPK may act antilipolytic and inhibit hormone sensitive lipase transport to the lipid droplet [54], which is in line with our observation of increasing ADIPOR1 mRNA abundance in AT. Therefore, AMPK may protect the adipocyte against energy depletion as discussed by Gauthier et al. [55]. Antilipolytic effects of AMPK were also addressed for dairy cows based on increasing phosphorylation of AMPKα1 after parturition [56]. Summarising, increasing ADIPOR1 mRNA throughout lactation might be associated with reduced lipolysis in dairy cows by increasing AMPK activity which would be in line with positive effects of ADIPOQ on lipid accumulation in AT [45] and lowest ADIPOQ concentrations after parturition in dairy cows [40].

Gene expression of PPARγ and PPARγ2 throughout lactation

The transcription factor PPARγ is a key regulator of IS and adipogenesis [57]. Targeted deletion of PPARγ in murine liver decreased hepatic triglyceride content and impaired systemic IS [58]. Therefore, regulation of PPARγ around parturition may affect insulin responsiveness of bovine tissues. Like in other species, t*wo* PPAR*γ* isoforms are defined in cattle i.e. PPARγ1 and PPARγ2 [14]. In vitro, the isoform PPARγ2 has stronger adipogenic activity compared to the isoform PPARγ1 [59].

In the pluriparous cows of Trial 1, PPARγ was reduced in s.c. AT and in the liver after parturition. Due to technical reasons which might be related to the amount of total RNA used for cDNA synthesis it was not possible to quantify PPARγ2 mRNA abundance in liver of all samples but we observed a curve shape comparable to PPARγ in s.c. AT. We suggest that the expression patterns of PPARγ and PPARγ2 mRNA, with highest values a.p. compared to postpartum in AT, are possibly associated to other genes we analysed throughout lactation. One example might be the regulation of ADIPOQ whose gene expression and plasma concentrations are induced by PPARγ ligands [60]. Lower values in PPARγ mRNA abundance in liver were also reported 3 wk after parturition as compared to the first wk of lactation [61]. Therefore, we expect a nadir of PPARγ expression in AT but also in liver in wk 3 postpartum. This observation is in contrast to PPARα which is upregulated after parturition [62]. As discussed by Lee et al. [63], the uppermost expression for PPARγ is found in AT, where it is related to adipogenesis and IS. In liver, it is linked to fatty acid uptake, lipid storage, and reduced gluconeogenesis. High abundance of PPARγ mRNA in s.c. AT during late pregnancy might be related to energy accretion by AT. In liver, the decreased abundance of PPARγ during early lactation may result in increased gluconeogenic capacity.

In Trial 2, no changes in PPARγ and PPARγ2 were observed in s.c. AT after parturition, which is in line with the results obtained in s.c. AT from Trial 1. In liver of the primiparous cows, both transcripts were increased with DIM and are thus in line with our observations in liver of the pluriparous cows from Trial 1. In addition to the potential effects of PPARγ on gluconeogenesis, the incidence for fatty liver might be reduced by decreasing expression of PPARγ2 after parturition: inactivation of PPARγ2 may improve fatty liver induced by high fat diet in mice [64].

For the mammary gland, Bionaz and Loor [65] reported that PPARγ is upregulated at the onset of lactation in comparison to 15 DIM. We analysed PPARγ2, which was not

regulated throughout lactation in mammary gland. Also in another study, no differences for PPARγ2 were observed in this tissue [66].

Gene expression of the pro-inflammatory proteins IL-6 and TNF-α

The adipokines IL-6 and TNF-α are related to insulin resistance [67]. In dairy cows, plasma TNF-α is reduced after parturition [68] and high TNF-α serum concentrations are associated with insulin resistance and fatty liver 1 to 2 wk after calving [69]. For serum IL-6, no differences were observed at different stages of lactation [70]. Limited data is available about the mRNA expression of both adipokines in bovine AT, but liver TNF-α mRNA abundance is higher compared to the one of IL-6 [71].

In Trial 1, we observed no changes of TNF-α mRNA throughout lactation in liver and in s.c. AT thus confirming earlier reports focusing on the transition period [61,72] and also extending the data base to the following periods of lactation. In contrast, IL-6 mRNA was reduced in liver during the transition period. IL-6 not only has adverse effects on liver metabolism but also stimulates proliferation and prevents hepatocyte damage [73]. The reduction around parturition might be related to a higher susceptibility for metabolic health disorders during early lactation [74]. The abundance of IL-6 mRNA in s.c. AT of pluriparous cows was too low for valid quantification by our protocol and was not detectable in each sample. By our data we were not able to confirm quantitative data on TNF-α mRNA from another study in pluriparous dairy cows where two peak were observed at 1 and 49 DIM [62].

In primiparous cows (Trial 2), we observed an increase in TNF-α mRNA abundance in all s.c. AT, at least in one of the sampling dates after parturition, which is different from the studies of Sadri et al. [72] and van Dorland et al. [61] reporting no changes during early lactation in pluriparous cows. In retroperitoneal AT, TNF-α mRNA was less abundant at 105 than on 1 DIM. In contrast to pluriparous cows, we observed an increase in the TNF-α mRNA abundance in liver. Therefore, we suggest that differential expression of TNF-α in primiparous vs. pluriparous cows and between different AT of primiparous cows occurs. Differences between the different AT depots might be related to local regulation of IS in either a paracrine or autocrine manner.

As discussed by Alluwaimi [75], TNF-α is suggested to maintain and regulate immunological function within the mammary gland and its expression is elevated throughout lactation, with a further increase in late vs. mid-lactation. In contrast, we observed a decrease of TNF-α mRNA in the mammary gland of primiparous cows during

the first 105 DIM.

The IL-6 mRNA in s.c. AT from tail head and withers was reduced from 1 to 105 DIM. Therefore, the regulation of the pro-inflammatory proteins IL-6 and TNF-α seems different at least at the level of the mRNA in these AT depots. In contrast, the IL-6 mRNA in liver was increased during this time. This observation might be related to an improved protective and regenerative capacity related to IL-6 at this stage of lactation [73] in primiparous, and, to a lower extent, in pluriparous cows, where only a numerical increase was observed. On the other hand, IL-6 is associated with reduced IS [67]. Network analysis in cows revealed that IL-6 is related to many liver specific pathways e.g. lipoprotein metabolism and fatty acid oxidation and increases during ketosis [76]. Therefore, lower abundance in liver around parturition and early lactation might be advantageous in relation to IS but the protection of the liver against oxidative stress during this time period might be reduced. This assumption is based on the positive effects of IL-6 regarding to liver cell damage and proliferation [73].

Effects of CLA supplementation on gene expression

In general, effects of CLA supplementation were not consistent between genes, tissues and time points throughout lactation. Effects of CLA were only observed in few cases, in which the abundance of the respective target mRNA was always decreased, with only one exception (LEPRB in liver of pluriparous cows). The ADIPOQ mRNA was reduced in omental and the ADIPOR2 mRNA in retroperitoneal AT at 105 DIM. In addition, lower TNF-α mRNA abundance was observed in mesenteric AT of the CLA group, although only at 42 DIM. Comparable to a study of Sigl et al. [77] using primiparous Brown Swiss cows, we detected no CLA effect on PPARγ mRNA abundance in liver of primiparous German Holstein cows but in pluriparous animals. CLA are ligands for PPARs [17,18]. Nevertheless, the effects of CLA in primiparous dairy cattle may differ from the effects of the PPARγ agonist 2,4-thiazolidinedione observed in bulls where a decrease of PPARγ mRNA expression was detected in liver, after treatment with this compound [78]. However, the effects of 2,4-thiazolidinedione were not consistent in bulls comparable to our CLA effects observed in primiparous and pluriparous cows. In bulls, PPARγ mRNA decreased by treatment with 2,4-thiazolidinedione in liver, increased in s.c. AT as a trend but had no effect in muscle. Therefore, inconsistency for PPARγ agonists like 2,4-thiazolidinedione and CLA between different tissues or AT depots and parity in cattle may exists, which is different to mice, were broad consistency was observed regarding to

the effects of both compounds on glucose and lipid metabolism [79].

For the mammary gland, we suggest that the impact of CLA supplementation is most important compared to all other analysed tissues. Treatment with CLA decreased PPARγ2 mRNA at both time points investigated in primiparous cows, accounting for 2 of 5 CLA effects we observed in total. PPARγ agonists have been demonstrated to stimulate lipid synthesis in a mammary epithelial cell line [12]. We observed lower PPARγ2 mRNA abundance at 42 and at 105 DIM in the animals treated with CLA as compared to the cows receiving the control fat supplement. The mammary gland is an important target for CLA; as discussed by Bauman et al. [22], milk fat depression by CLA treatment is well established. PPARγ was suggested by Kadegowda et al. [12] to be an important regulator of milk fat synthesis. It was shown that in parallel to PPARγ, lipogenic genes like stearoyl-CoA desaturase, diacylglycerol *O*-acyltransferase 2 and fatty acid synthase were down regulated during the peripartal period [80]. We suggest that reduced receptor capacity of the PPARγ2 isoform might be responsible for milk fat depression by CLA.

CLA supplementation and systemic insulin sensitivity

In pluriparous cows, no CLA effects were observed on NEFA and BHBA concentrations [81]. Nevertheless, different to primiparous cows of Trial 1, insulin and glucose concentrations were higher and the RQUICKI index was reduced by 8 % during the supplementation period and by 17 % during the depletion period after 182 DIM in CLA treated cows vs. control cows. This indicates a CLA-induced reduction of IS in pluriparous cows. Comparable effects on insulin concentrations using a similar CLA mixture were also observed in a mouse model, in which the results were explained by an increase in ß-cell mass and number [82]. In contrast to our results, Hötger et al. [23] observed an increase of plasma glucose by CLA treatment in dairy cows during second lactation but not of insulin which might be related to the shorter supplementation period of 9 weeks compared to 26 weeks in our study. Insulin is a central regulator of energy metabolism and different responses to CLA between primiparous and pluriparous cows might be related to differences in basal insulin secretion which may influence the pancreatic response capacity to CLA treatment. Primiparous cows have higher insulin concentrations than pluriparous cows during the first 50 DIM [83], comparable to our data up to 182 DIM. Recently, it was shown that CLA is a ligand for the free fatty acid receptor 1 but only the *trans*-10, *cis*-12 in contrast to the *cis*-9, *trans*-11 CLA isomer increases glucose stimulated insulin secretion via this receptor [20]. Differences in its

expression in pancreatic ß-cells between primiparous vs. pluriparous cows might be interesting to explore in future as information on this issue may help to explain differences between these two groups.

However, albeit the effects of CLA treatment on IS were significant, it remains open whether this might imply adverse effects on animal health. The values recorded herein to characterise IS were comparable to earlier reports [84], but currently no reference values for healthy versus diseased cows are available to allow for a final judgment.

Conclusion

Studying primiparous and pluriparous cows, we observed changes in the mRNA abundance of different modulators of IS like the ADIPOQ and LEP system in different tissues focusing on AT and liver. Differences between different AT depots, even of s.c. origin should be considered for further studies. Our results revealed few individual differences between primiparous and pluriparous cows. Potential insulin desensitizing effects of CLA supplementation, as reported in monogastric animals, were observed in few selected tissues of dairy cows but there was no consistency regarding to the analysed parameters. Supplementation of cows with CLA reduced systemic IS significantly in pluriparous cows but not in primiparous cows. Finally, we showed that PPARγ2, as a regulator of milk fat synthesis in cattle, is reduced in mammary gland by CLA supplementation which might be a direct link to CLA induced milk fat depression.

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Authors contributions

Conceived and designed the experiments: SD, GB, JR, HS, MM. Performed the experiments: BS, SW, PF, AK, JR, MM, SH. Analysed the data: BS, MM. Wrote the paper: BS, MM, HS.

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Table 1. Characteristics of the primers and the real-time PCR conditions

Primers	Sequence (5'-3')	NIH Genbank accession number	bp[11]	Concen-tration (nmol)	Mean Cq[12] value[13]	Annealing[14] (sec/°C)
ADIPOQ[1]						
Forward	CTGGAGAGAAGGGAGAGAAAG	NM_174742	204	800	24.9	75/60
Reverse	TGGGTACATTGGGAACAGTG					
ADIPOR1[2]						
Forward	GCTGAAGTGAGAGGAAGAGTC	XM_593692	118	800	22.3	45/60
Reverse	GAGGGAATGGAGTTTATTGCC					
ADIPOR2[3]						
Forward	GGCAACATCTGGACACATC	XM_580459	200	400	21.5	45/60
Reverse	CTGGAGACCCCTTCTGAG					
LEP[4]						
Forward	GACATCTCACACACGCAG	U62123	183	400	25.4	30/60
Reverse	GAGGTTCTCCAGGTCATT					
LEPRB[5]						
Forward	ACCACACCTTCCGTTCTCAG	AB199589	164	400	28.8	30/60
Reverse	GGGACAACACTCTTGACTC					
LEPR[6]						
Forward	CCACTGTTGCTTTTGGAGCGAGGA	NM_001012285	125	100	23.0[15]	60/62
Reverse	TGTTCCAGTTTGCACCTGTTTGCT					
PPARγ2[7]						
Forward	ATTGGTGCGTTCCCAAGTTT	Y12420	57	400	21.0	60/60
Reverse	GGCCAGTTCCGTTCAAAGAA					
PPARγ[8]						
Forward	AGGATGGGGTCCTCATATCC	Y12420	121	800	25.3	60/61
Reverse	GCGTTGAACTTCACAGCAAA					
IL-6[9]						
Forward	TGCAGTCTTCAAACGAGTGG	BC123577	182	400	26.6	60/30
Reverse	TAAGTTGTGTGCCCAGTGGA					
TNF-α[10]						
Forward	TGCCTGCTGCACTTCGGGGTA	EU276079	50	800	28.8	60/60
Reverse	CCTGGGGACTCTTCCCTCTGGGG					

[1] Adiponectin, [42]
[2] Adiponectin receptor 1, [42]
[3] Adiponectin receptor 2, [42]
[4] Leptin, [85]
[5] Leptin receptor isoform b, [42]
[6] Leptin receptor
[7] Peroxisome proliferator-activated receptor *gamma2*
[8] Peroxisome proliferator-activated receptor *gamma*, [86]
[9] Interleukin-6
[10] Tumor necrosis factor-α
[11] bp: base pairs.
[12] Cq: Quantification cycle.
[13] Based on slaughter experiment.
[14] Initial denaturation = 10 min at 95°C; denaturation = 30 s at 95°C; extension = 30 s at 72°C, except for *TNF-α*, *PPARγ2*, *PPARγ*, *LEPR*, and *LEPRB* (60 s at 72°C).
[15] Based on liver and s.c. fat biopsies from Trial 1.

Table 2. *ADIPOQ* system and Peroxisome proliferator-activated receptor γ and γ2 mRNA expression in different tissues of primiparous cows supplemented with or without CLA in a time course of 105 days in milk (DIM)

Gene	DIM	Treatment	Tissue mRNA abundance (arbitrary unit)								
			Omental	Mesenteric	Retroperitoneal	s.c. tail head	s.c. withers	s.c. sternum	Muscle[1]	Mammary[2]	Liver
ADIPOQ[3]	1	Control	0.63±0.20^{a}	0.69 ± 0.31	1.04±0.24^{a}	1.16±0.15	1.62±0.52	0.87±0.23		0.10±0.02	
	42	Control	0.89±0.36^{a}	0.41±0.12	1.05±0.20^{a}	1.24±0.28	1.08±0.68	0.68±0.21		0.16±0.05	
	42	CLA	1.35±0.41	0.62±0.37	1.22±0.40^{a}	0.90±0.63	1.13±0.48	0.61±0.21		0.42±0.26	
	105	Control	**2.11±0.51**^{b}	1.14±0.58	2.86±0.49^{b}	1.91±0.39	1.16±0.47	0.53±0.27		0.20±0.04	
	105	CLA	**0.83±0.44**	0.88±0.35	1.71±0.46^{b}	1.22±0.28	1.66±0.38	1.09±0.26		0.30±0.09	
ADIPOR1[4]	1	Control	0.80±0.05	0.60±0.06^{a}	0.80±0.09^{ab}	0.85±0.04^{a}	0.89±0.06^{a}	0.84±0.05^{a}	1.69±0.14^{a}	0.89±0.05^{a}	0.87±0.06
	42	Control	0.87±0.18	0.62±0.03^{a}	0.88±0.06^{a}	0.89±0.14^{a}	0.80±0.13	0.84±0.06^{a}	0.94±0.06^{b}	0.91±0.07^{a}	0.74±0.05^{a}
	42	CLA	1.08±0.12	0.67±0.05^{a}	0.79±0.04^{a}	0.79±0.12	1.35±0.26	0.83±0.08^{a}	1.06±0.10^{b}	0.92±0.11^{a}	0.75±0.07^{a}
	105	Control	1.07±0.12	0.88±0.15^{b}	1.18±0.11^{b}	1.34±0.16^{b}	1.28±0.17^{b}	1.07±0.09^{b}	1.06±0.05^{b}	1.15±0.08^{b}	0.87±0.03^{b}
	105	CLA	0.85±0.18	0.76±0.05^{b}	1.03±0.13^{b}	1.06±0.14	1.28±0.15^{b}	1.20±0.13^{b}	0.93±0.05^{b}	1.15±0.05^{b}	0.88±0.04^{b}
ADIPOR2[5]	1	Control	1.70±0.60	1.97±0.70	3.65±0.80	3.23±0.76	4.51±1.64	2.64±0.90	1.35±0.08	1.43±0.53	1.62±0.39
	42	Control	1.87±0.86	1.39±0.40	3.22±0.42^{a}	3.01±1.04	2.87±2.55	1.81±0.82	1.19±0.18	0.77±0.17^{a}	1.44±0.32
	42	CLA	3.03±1.24	1.92±1.03	3.21±1.04^{a}	2.68±2.04	2.88±1.38	2.32±0.86	0.99±0.08	0.95±0.30^{a}	1.56±0.30
	105	Control	3.68±1.32	2.21±1.01	**5.94±1.31**^{b}	5.21±1.48	3.69±2.06	1.98±1.15	1.42±0.10	2.56±0.61^{b}	1.29±0.15
	105	CLA	2.02±1.02	1.37±0.57	**2.56±0.70**^{b}	2.18±0.71	2.56±1.23	2.81±1.18	1.22±0.11	1.86±0.16^{b}	1.06±0.05
PPARγ[6]	1	Control	1.18±0.13^{a}	1.58±0.27	1.76±0.30	1.97±0.32	2.34±0.26	1.57±0.28			0.65±0.11^{a}
	42	Control	1.50±0.45^{ab}	1.57±0.28	2.94±0.35	2.45±0.50	1.57±0.48	1.74±0.17			0.78±0.05^{ab}
	42	CLA	2.04±0.34^{ab}	1.92±0.24	2.45±0.30	1.59±0.22	1.82±0.42	1.29±0.34			0.76±0.07^{ab}
	105	Control	1.98±0.45^{b}	2.48±0.42	3.57±0.49	2.56±0.47	2.05±0.51	1.67±0.27			0.85±0.11^{b}
	105	CLA	2.47±0.45^{b}	3.25±0.66	3.32±0.78	2.53±0.53	2.99±0.52	2.34±0.38			1.10±0.12^{b}
PPARγ2[7]	1	Control	0.88±0.15	0.87±0.25	0.92±0.25^{a}	1.47±0.16	1.78±0.45	0.83±0.23	0.69±0.13	1.15±0.39	0.56±0.06^{a}
	42	Control	1.42±0.47	0.76±0.09	1.33±0.21^{ab}	1.71±0.49	0.80±0.39	0.91±0.16	0.82±0.04	**1.15±0.23**	0.68±0.08^{a}
	42	CLA	1.76±0.32	1.14±0.25	1.19±0.06^{ab}	1.13±0.31	1.17±0.31	0.62±0.14	0.82±0.16	**0.68±0.05**	0.65±0.08^{a}
	105	Control	1.47±0.37	1.29±0.23	1.59±0.13^{b}	1.59±0.23	0.97±0.26	0.74±0.06	0.71±0.09	**0.97±0.14**	0.98±0.25^{b}
	105	CLA	1.24±0.33	1.11±0.20	1.45±0.24^{b}	1.55±0.36	1.57±0.21	1.13±0.15	0.78±0.12	**0.75±0.12**	1.23±0.21^{b}

CLA: Lutrell® Pure, BASF SE, Ludwigshafen, Germany. Control: Silafat®, BASF SE.

Significant differences ($P < 0.05$) between different days per tissue are defined using different letters. Significant differences ($P < 0.05$) between CLA and control group within day and tissue are depicted by bold numbers.

Data are normalized based on the geometric mean of Eukariotic translation initiation factor 3 (EIF3K), Lipoprotein receptor-related protein 10 (LRP10), RNA polymerase II (POLR2A), Emerin (EMD), Marvel domain containing 1 (MARVELD1), and Hippocalcin-like 1 (HPCAL1) for each s.c. fat and mesenteric fat depots; EIF3K, LRP10, POLR2A, EMD, and MARVELD1 for omental and retroperitoneal fat depots; HPCAL1, LRP10, POLR2A, EIF3K, Glyceraldehyde-phosphate-dehydrogenase (GAPDH) for liver; LRP10, EMD, POLR2A, EIF3K for muscle, and MARVELD1, EMD, LRP10, EIF3K, POLR2A, HPCAL1 for mammary gland tissue.

[1] Semitendinosus. [2] *ADIPOQ* in mammary gland tissue was only detectable in one-third of the samples. [3] Adiponectin. [4] Adiponectin receptor 1. [5] Adiponectin receptor 2. [6] Peroxisome proliferator-activated receptor γ. [7] Peroxisome proliferator-activated receptor γ2; Means ± SE.

Table 3. *LEP* system, *IL-6,* and *TNF-α* mRNA expression in different tissues of primiparous cows supplemented with or without CLA in a time course of 105 DIM

Gene	DIM	Treatment	Tissue mRNA abundance (arbitrary unit)								
			Omental	Mesenteric	Retroperitoneal	s.c. tail head	s.c. withers	s.c. sternum	Muscle	Mammary	Liver
LEP[1]	1	Control	0.43±0.11	0.80±0.27	1.70±0.37	0.87±0.21	1.14±0.34	0.77±0.27	0.18±0.09	0.29±0.15	
	42	Control	0.89±0.38	0.67±0.25	1.07±0.38	1.01±0.39	0.73±0.67	1.24±0.60	0.18±0.08	0.35±0.10	
	42	CLA	1.67±0.57	0.88±0.30	1.44±0.55	0.92±0.61	1.85±1.10	1.63±0.83	0.71±0.28	0.43±0.17	
	105	Control	2.23±1.11	1.33±0.67	3.33±0.65	1.40±0.27	1.69±0.74	0.76±0.15	0.50±0.27	0.15±0.05	
	105	CLA	1.38±0.76	0.86±0.26	1.97±0.67	1.16±0.39	1.80±0.54	1.76±0.59	0.34±0.13	0.23±0.10	
LEPRB[2]	1	Control	1.57±0.41^{a}	0.67±0.19	1.60±1.06^{ab}	0.54±0.14	1.19±0.49	2.42±0.85	0.35±0.05	0.52±0.07^{a}	1.49±0.27
	42	Control	0.41±0.12^{b}	0.67±0.20^{a}	1.35±0.31^{a}	0.42±0.30	0.72±0.32	2.45±1.10	0.71±0.14	0.26±0.11^{ab}	1.29±0.14
	42	CLA	0.51±0.20^{b}	0.79±0.26^{a}	1.57±0.53^{a}	0.22±0.05	1.06±0.21	0.98±0.15	0.70±0.11	0.37±0.03^{ab}	1.98±0.41
	105	Control	0.97±0.67^{b}	0.27±0.11^{b}	0.54±0.17^{b}	0.27±0.10	0.44±0.14	1.14±0.23	0.59±0.10	0.17±0.11^{b}	1.20±0.18
	105	CLA	0.56±0.18^{b}	0.34±0.16^{b}	0.58±0.21^{b}	0.24±0.09	1.06±0.45	2.33±0.84	0.72±0.14	0.28±0.08^{b}	1.37±0.16
TNF-α[3]	1	Control	1.14±0.18	0.58±0.10	1.06±0.16	0.65±0.05^{a}	0.58±0.09^{a}	1.08±0.19^{a}	0.72±0.13	1.81±0.33^{a}	0.52±0.06^{a}
	42	Control	1.37±0.40	**1.00±0.10**	0.92±0.12^{a}	1.27±0.21^{b}	1.37±0.28^{b}	1.45±0.36^{ab}	1.03±0.16	0.81±0.14^{b}	1.12±0.20^{b}
	42	CLA	1.33±0.36	**0.72±0.25**	1.09±0.17^{a}	1.74±0.63^{b}	1.06±0.22^{b}	1.34±0.23^{ab}	1.08±0.38	0.96±0.12^{b}	1.42±0.11^{b}
	105	Control	0.83±0.27	0.84±0.11	0.64±0.16^{b}	1.03±0.37^{ab}	1.13±0.39^{ab}	2.25±0.53^{b}	0.92±0.12	0.99±0.14^{b}	1.62±0.21^{b}
	105	CLA	1.70±0.43	1.12±0.37	0.69±0.11^{b}	1.15±0.27^{ab}	1.01±0.12^{ab}	1.77±0.39^{b}	0.94±0.12	0.68±0.13^{b}	1.56±0.27^{b}
IL-6[4]	1	Control	2.77±1.57	0.85±0.41	1.43±0.36	4.30±2.29^{a}	7.31±2.07^{a}	0.52±0.13	0.85±0.20	0.68±0.14	0.36±0.08^{a}
	42	Control	0.82±0.09	0.68±0.08	0.88±0.31	1.62±0.40^{ab}	2.79±1.38^{b}	0.53±0.13	0.88±0.18	0.83±0.10	0.70±0.10^{b}
	42	CLA	0.74±0.06	0.79±0.31	0.70±0.07	1.38±0.55^{ab}	1.68±0.36^{b}	0.34±0.13	0.86±0.13	0.83±0.19	0.78±0.15^{b}
	105	Control	1.08±0.66	0.36±0.15	0.59±0.15	0.90±0.25^{b}	0.69±0.36^{b}	0.26±0.09	0.76±0.11	0.95±0.14	0.88±0.11^{b}
	105	CLA	3.98±1.37	1.65±0.16	2.13±1.41	3.65±2.53^{ab}	2.22±0.73^{b}	0.55±0.15	0.94±0.14	0.82±0.09	0.73±0.12^{b}

CLA: Lutrell® Pure, BASF SE, Ludwigshafen, Germany.
Control: Silafat®, BASF SE.
Significant differences between different days per tissue are defined using different letters. Significant differences ($P < 0.05$) between CLA and control group within day and tissue are depicted by bold numbers.
Data are normalized based on the geometric mean of Eukariotic translation initiation factor 3 (EIF3K), Lipoprotein receptor-related protein 10 (LRP10), RNA polymerase II (POLR2A), Emerin (EMD), Marvel domain
containing 1 (MARVELD1), and Hippocalcin-like 1 (HPCAL1) for each s.c. fat and mesenteric fat depots; EIF3K, LRP10, POLR2A, EMD, and MARVELD1 for omental and retroperitoneal fat depots; HPCAL1, LRP10, POLR2A, EIF3K, Glyceraldehyde-phosphate-dehydrogenase (GAPDH) for liver; LRP10, EMD, POLR2A, EIF3K for muscle, and MARVELD1, EMD, LRP10, EIF3K, POLR2A, HPCAL1 for mammary gland tissue.
[1] Leptin. [2] leptin receptor isoform b. [3] Tumor necrosis factor-α. [4] Interleukin-6; Means ± SE.

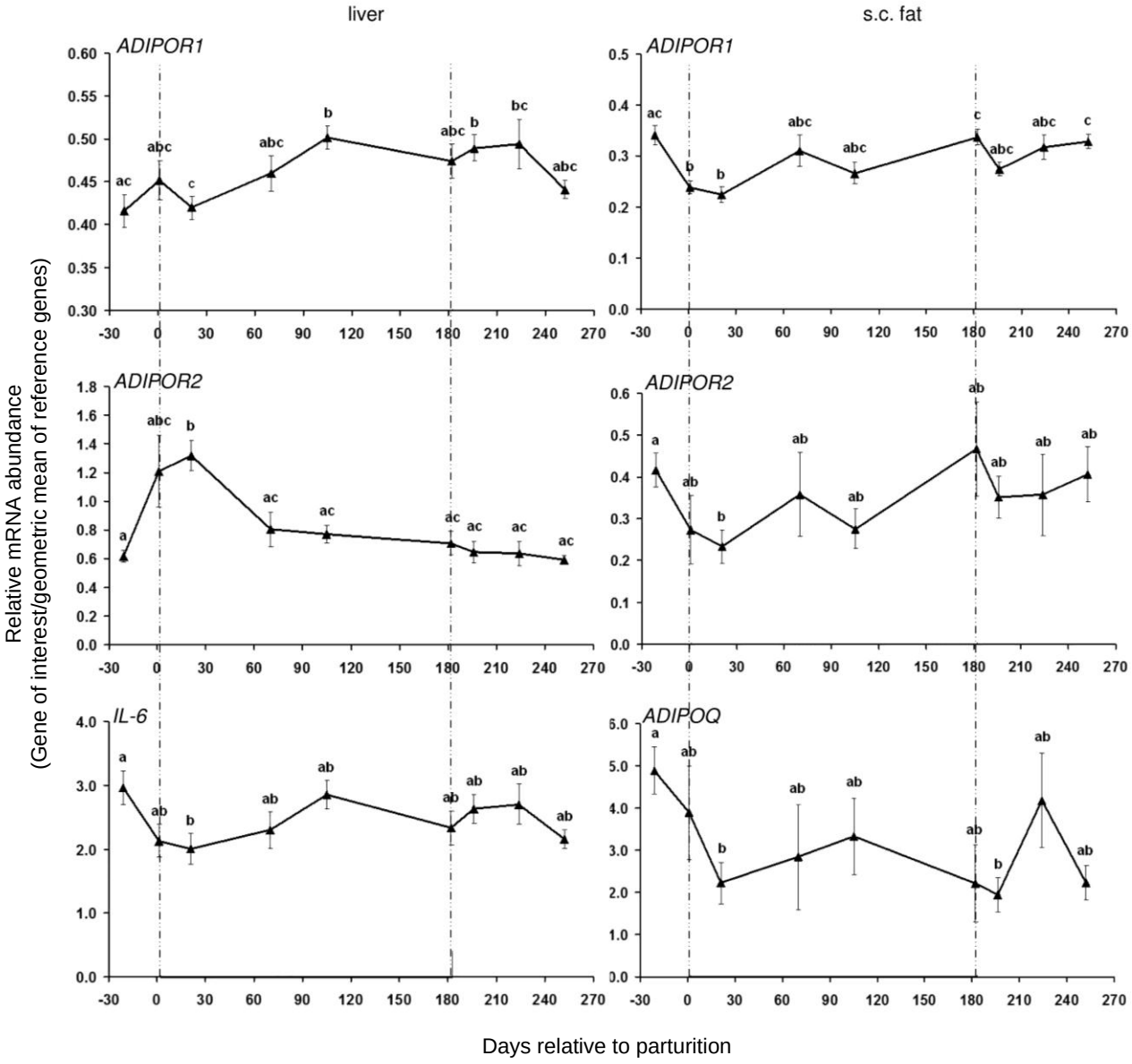

Figure 1. Longitudinal mRNA expression of adiponectin receptors (ADIPOR1 and ADIPOR2) in liver and s.c. tail head biopsies of dairy cows together with adiponectin (ADIPOQ) and interleukin-6 (IL-6) mRNA abundance in s.c. adipose tissue and in liver of Trial 1, respectively. Cows were fed with conjugated linoleic acids (CLA, Lutrell® Pure, BASF SE, Ludwigshafen, Germany) at 100 g/day CLA or a control fat supplement (Silafat®, BASF SE) from day 1 until day 182 postpartum in Trial 1. Primiparous cow samples are not included (There are no sample in the CLA group at days 1, 70, 182, and 224) [Control: n = 10, CLA: n = 11]. Cumulative mRNA expression of both control and CLA is shown because CLA effect was insignificant. For normalization, lipoprotein receptor-related protein 10 (LRP10), RNA Polymerase II (POLR2A) and eukariotic translation initiation factor 3 (EIF3K) in liver and LRP10, glyceraldehyde-phosphate-dehydrogenase (GAPDH), and POLR2A in s.c. adipose tissue were used as reference genes. Different letters indicate significant differences between days relative to parturition ($P < 0.05$; mean ± SEM). Area between vertical lines corresponds to the CLA supplementation period.

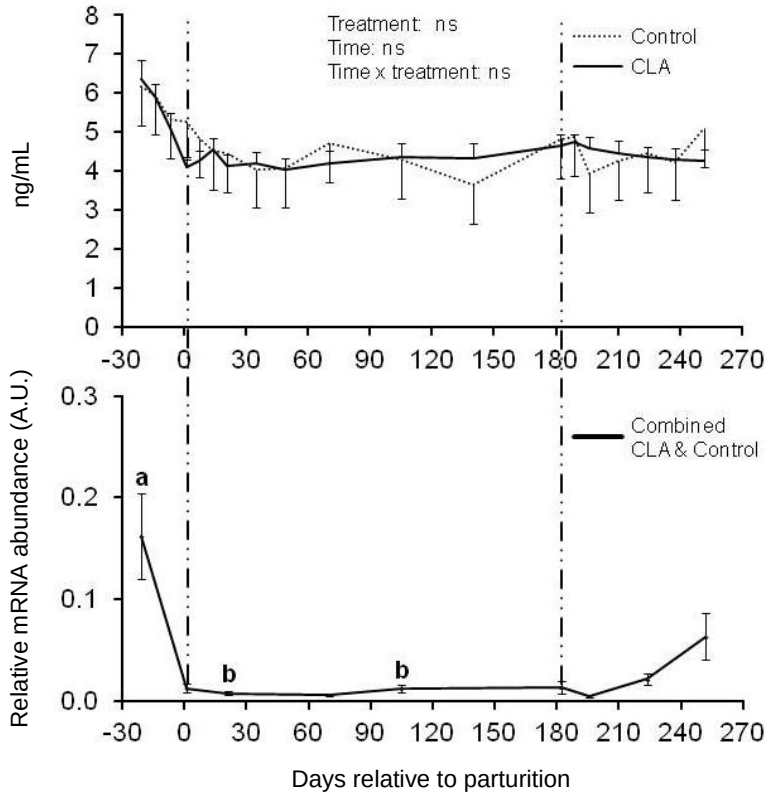

Figure 2. Leptin serum concentrations (upper graph) and LEP mRNA abundance in s.c. tail head adipose tissue (downer graph) in pluriparous and primiparous cows receiving conjugated linoleic acids (CLA, Lutrell® Pure, BASF SE, Ludwigshafen, Germany) at 100 g/day (CLA) or a control fat supplement (Silafat®, BASF SE) from day 1 until day 182 postpartum in Trial 1. All animals and samples were included for serum data [Control: pluriparous cows n = 10, primiparous cows n = 5; CLA: pluriparous cows n = 11, primiparous cows n = 5]. For mRNA data only pluriparous cow samples from days -21, 1, 21, 70, 105 182 196, 224, and 252 relative to parturition for control group and days -21, 21, 105, 196, and 252 for CLA group were analysed. For normalization, lipoprotein receptor-related protein 10 (LRP10), RNA Polymerase II (POLR2A) and glyceraldehyde-phosphate-dehydrogenase (GAPDH) in s.c. adipose tissue were used as reference genes. ns: not significant. Different letters indicate significant differences between days relative to parturition ($P < 0.05$; mean ± SEM). Area between vertical lines corresponds to the CLA supplementation period. A.U.: arbitrary units.

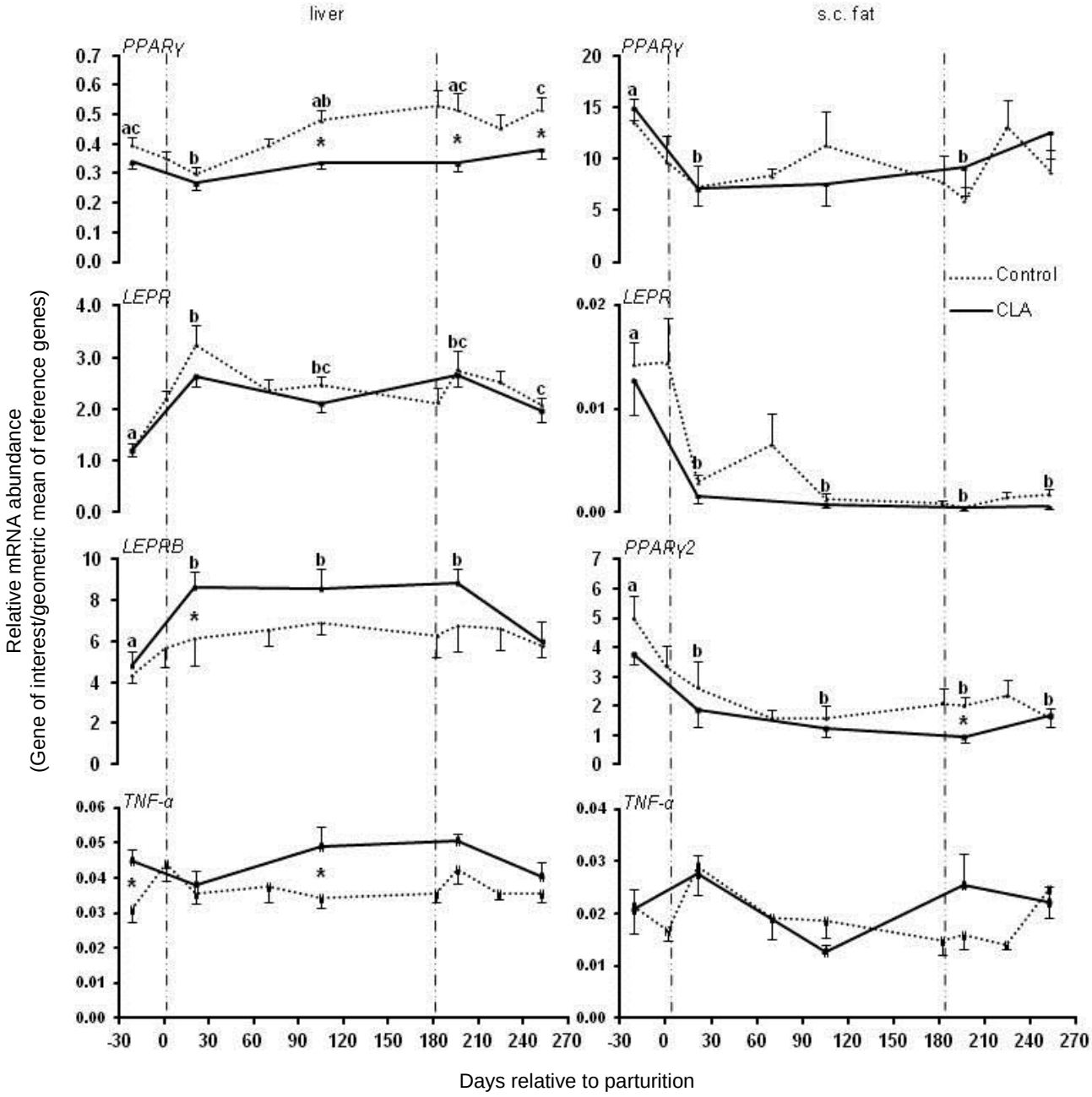

Figure 3. Longitudinal mRNA expression of peroxisome proliferator-activated receptor (PPAR) *γ* and *γ2*, leptin receptor (LEPR), leptin receptor isoform b (LEPRB), and tumor necrosis factor (TNF)-*α* in liver and s.c. tail head adipose tissue biopsies of dairy pluriparous cows fed with conjugated linoleic acids (CLA, Lutrell® Pure, BASF SE, Ludwigshafen, Germany) at 100 g/day CLA or a control fat supplement (Silafat®, BASF SE) from day 1 until day 182 postpartum in Trial 1(defined by vertical lines). Primiparous cow samples are not included. There are no sample in the CLA group at days 1, 70, 182, and 224 [Control: n = 10, CLA: n = 11]. For normalization, lipoprotein receptor-related protein 10 (LRP10), RNA Polymerase II (POLR2A) and eukariotic translation initiation factor 3 (EIF3K) in liver and LRP10, glyceraldehyde-phosphate-dehydrogenase (GAPDH), and POLR2A in s.c. adipose tissue were used as reference genes. Different letters indicate significant differences between days relative to parturition where there are differences otherwise no letter used. CLA effects are defined using asterisks ($P < 0.05$; mean ± SEM). Area between vertical lines corresponds to the CLA supplementation period.

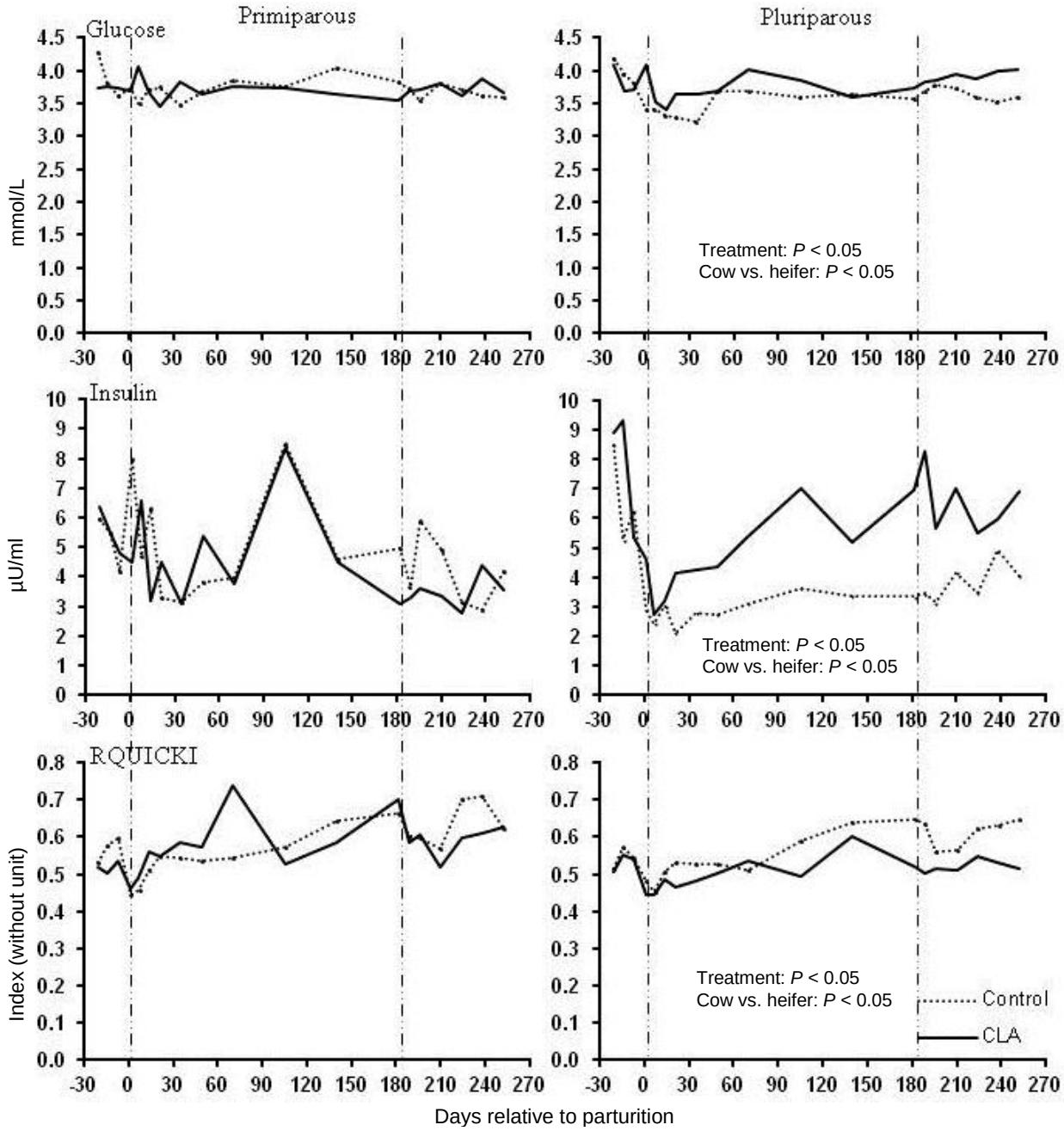

Figure 4. Glucose and insulin concentration in serum and calculated Revised Quantitative Insulin Sensitivity Check Index (RQUICKI) in cows receiving conjugated linoleic acids (CLA, Lutrell® Pure, BASF SE, Ludwigshafen, Germany) at 100 g/day or a control fat supplement (Silafat®, BASF SE) from day 1 until day 182 postpartum [pluriparous cows (Control, n = 10 and CLA, n = 11) and primiparous cows (Control, n = 4 and CLA, n = 5)] in Trial 1. RQUICKI = 1/[log(glucose) + log(insulin) + log(NEFA)], [24]. Supplementation with CLA did not interfere with the parameters measured in primiparous cows. Area between vertical lines corresponds to the CLA supplementation period. Pooled SE for glucose, insulin, and RQUICKI were 0.21, 1.70, and 0.05 for heifers and 0.24, 1.86, and 0.05 for cows, respectively.

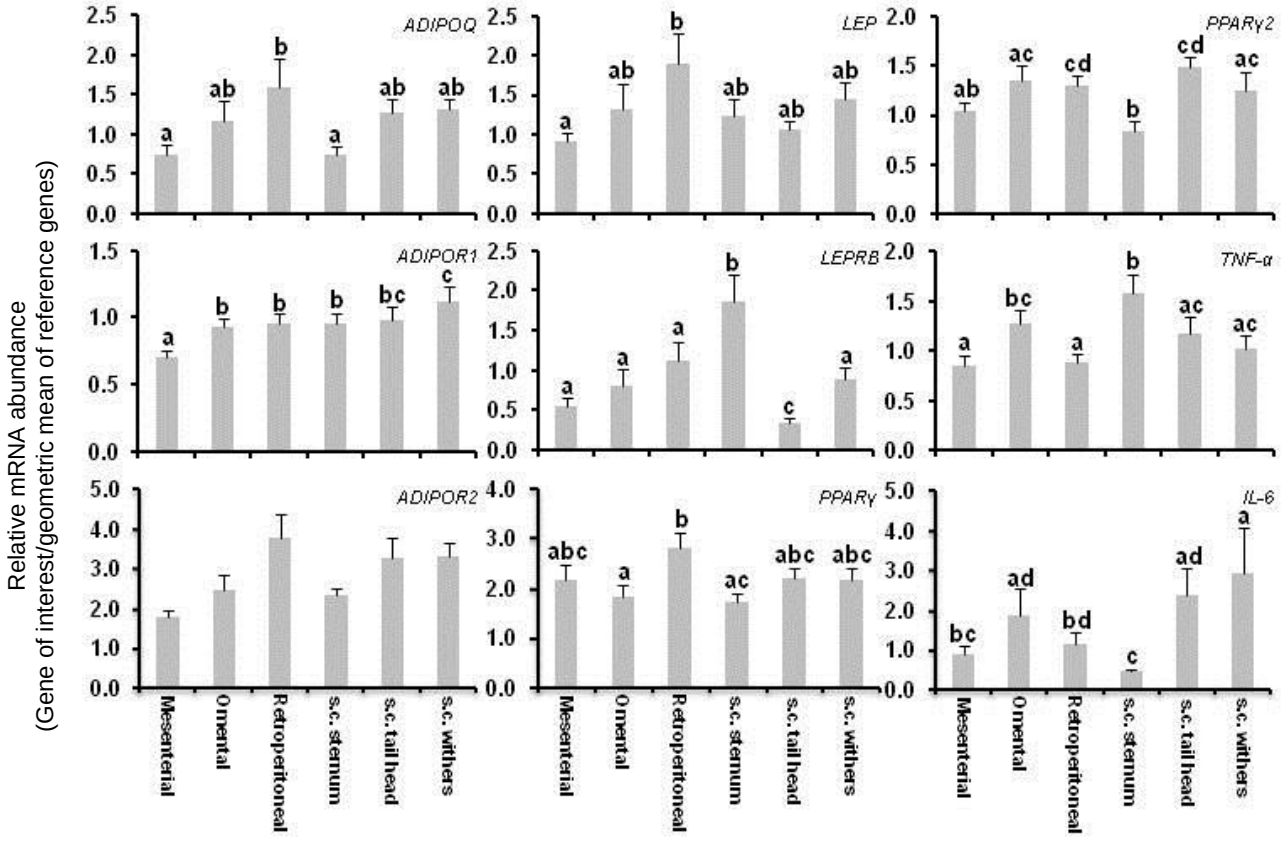

Figure 5. Individual fat depot differences of adiponectin (ADIPOQ), adiponectin receptors (ADIPOR1 and ADIPOR2), leptin (LEP), leptin receptor isoform b (LEPRB), peroxisome proliferator-activated receptor (PPAR) *γ* and *γ2*, tumor necrosis factor (TNF)-*α*, and interleukin (IL)-*6* mRNA abundance [mean of d 1 (n = 5), 42 (n = 10), and 105 (n = 10) post partum from primiparous cows (Trial 2) fed at 100 g/day with conjugated linoleic acids (CLA, Lutrell® Pure, BASF SE, Ludwigshafen, Germany) or a control fat supplement (Silafat®, BASF SE)] in Trial 2. Different letters specify the differences within fat depots ($P < 0.05$). Data are normalized based on the geometric mean of eukariotic translation initiation factor 3 (EIF3K), lipoprotein receptor-related protein 10 (LRP10), RNA polymerase II (POLR2A), emerin (EMD), marvel domain containing 1 (MARVELD1), and hippocalcin-like 1 (HPCAL1) for each s.c. fat and mesenteric fat depots and EIF3K, LRP10, POLR2A, EMD, and MARVELD1 for omental and retroperitoneal fat depots.

7. General discussion and conclusions

Conjugated linoleic acids are well known for their milk fat reducing effects in dairy cows. In the animal experiments studied herein, the efficacy of the CLA treatment was confirmed in this respect, i.e. milk fat percentage in both primi- and pluriparous cows was 14% and 12% less than in the corresponding control groups, respectively (von Soosten et al., 2011; Pappritz et al., 2011a). This effect of CLA was evident 42 and 28 days after onset of the CLA treatment in primiparous and pluriparous cows, respectively. However, most of the CLA effects on tissues investigated herein were only seen later in lactation. CLA treated primiparous cows had less back fat than their control counterparts (von Soosten et al., 2011) and the decrease of adipocyte size was observed in 4 out of 6 different fat depots in the CLA group at 105 days of supplementation, whereas fat cell size at 42 days was smaller in only 2 depots (Akter et al., 2011). Therefore, the time of CLA treatment seems to be an important determinant for the different effects. In addition, for CLA effects at the level of tissues, the dosage and the availability of the CLA isomers in intermediary metabolism is crucial. For the investigations of the present thesis, 100 g of commercially available CLA supplement were tested. For this supplement, a 10% content of each the *cis*-9, *trans*-11 and the *trans*-10, *cis*-12 CLA isomer and protection from ruminal degradation are claimed. In previous experiments feeding 25, 50 or 100 g of this supplement per day, the portion of *trans*-10, *cis*-12 isomer of total fatty acids from milk fat was 3 (0.03%), 6 (0.06%) and 12 fold higher (0.12%) than in control animals receiving no CLA (0.01%). For the *cis*-9, *trans*-11 isomer, the increase was less pronounced, i.e. 1.2 (0.55%), 1.4 (0.66%) and 1.9 fold (0.86%) higher, respectively (Brömmel et al., 2007; Meyer et al., 2007). Thus a significant portion of the consumed CLA reached the circulation and finally the mammary gland. In a more detailed approach using cows fitted with rumen and duodenal cannula, the portion of CLA escaping rumen biohydrogenation was determined to be between 5 and 16% (Pappritz et al., 2011b) and is thus comparable with other studies reporting 9-34% (de Veth et al., 2005). The CLA that reach the duodenum is mostly secreted into milk (36-48%), or is excreted via faeces (~ 50%) and thus only a small portion, i.e. 2-14% of the CLA in the duodenum would be able to reach different tissues and cells (Pappritz et al., 2011b). However, when comparing the absolute and relative concentrations of the two main CLA isomers in the two animal experiments investigated herein, distinctively lower concentrations were observed: in the trial from which we used the pluriparous cows, the percentage of the *cis*-

9, *trans*-11 isomer in milk fat (0.59%) was unchanged at 100 g/day vs. no CLA supplement (0.57%); for the *trans*-10, *cis*-12 CLA a 7.5 fold higher percentage (0.03%) than in the control (0.004%) was observed during the supplementation period (Pappritz et al., 2011a), i.e. only half of the difference reached in the earlier studies. Moreover, when comparing the percentage values from this with the earlier study, the content of *trans*-10, *cis*-12 CLA in milk fat was also less, i.e. 0.03% vs. 0.12%. Adipose tissue analyses for CLA contents were done for the primiparous cows from the present investigations (von Soosten, 2012): comparing 3 v.c. and the s.c. fat depot, the *cis*-9, *trans*-11 isomer concentrations were not affected by CLA treatment. For the *trans*-10, *cis*-12 CLA, significantly higher concentrations were reached in 2 v.c. and in the s.c. fat depot of CLA vs. control cows. However, the concentrations were very low and the significant difference was attributable to the fact that the portion of *trans*-10, *cis*-12 CLA in fat was below the limit of detection (<0.01% of total fatty acids) whereas the positive tissues had maximally 0.02%. In skeletal muscle tissue, CLA were not detectable. Overall, the transfer efficiencies of the consumed CLA into AT were very low, i.e. < 0.1%. Comparing the tissue concentration after 42 vs. 105 days of supplementation, slightly higher transfer efficiencies (0.18%) were observed with the 105 day treatment period. The low tissue concentrations reached in the current trials might thus be a reason for the rare and only scattered CLA effects observed in general in these two trials. When considering the modes of action of CLA, the focus in our works was on receptor-triggered pathways. An additional mechanism on how CLA may affect cells might also be of importance although it was beyond the scope of this thesis: CLA have been suggested to alter the basic properties of cell membranes when build into the lipid bilayer. As other polyunsaturated fatty acids, CLA seem sterically incompatible with sphingolipid and cholesterol; therefore, CLA appear to alter the biochemical make up of lipid rafts/caveolae microdomains, thereby influencing cell signaling, protein trafficking and cell cytokinetics (Chapkin et al., 2008). Indeed, in a recent study (Subbaiah et al., 2011) *trans*-10, *cis*-12 CLA was demonstrated to increase the cholesterol content of the raft fraction in cell culture studies. Moreover, very recently the incorporation of *cis*-9, *trans*-11 CLA in membranes of cancer cells was shown to make the cells more susceptible to X-radiation and thus points to functional importance of the membrane function altering effects of CLA, in particular in the raft-associated proteins (Grądzka et al., 2013). As pointed out above, incorporation specifically in cell membranes was not assessable in the present cow studies. However, in the frame of the cooperative project herein, the fatty

acid composition of the lipid fraction of blood cells (that have very low intracellular lipid concentrations) was determined. The portion of *trans*-10, *cis*-12 CLA in total fatty acids was increased (Renner et al., 2012) although only to a similarly low extent as seen for AT (i.e. from undetectable portions in the controls to 0.02% of total fatty acids). For comparison: in the cancer cells from the aforementioned study (Grądzka et al., 2013), the *cis*-9, *trans*-11 CLA portion was 20% of total fatty acids. The CLA dosages commonly used in monogastric species are generally much higher that for dairy cows. The milk fat reducing effect in dairy cows is the main target trait and for this, the relatively low dosage is - as manifold proven including the current studies - effective. There is no conclusive explanation why the tissue concentrations reached in our studies were lower than in previous studies, albeit the same CLA preparation was used. Nevertheless, the evidence for the milk fat reduction observed in the current studies demonstrates that active tissue concentrations were indeed achieved. Future studies using higher dosages or possibly a more efficient mode of rumen protection to increase the amount of CLA available in the small intestine might yield more pronounced effects on the variables tested herein. In addition, the situation might be different if the CLA treatment would have been started already during late pregnancy as compared to the first day of lactation in the current studies. Moreover, in view of the fact that some of the effects of CLA i.e. increased insulin concentration, reduced insulin sensitivity and reduced *PPARγ* mRNA abundance in liver tissue were maintained beyond the actual time of CLA treatment, potential effects on cell membrane traits are supported, since both incorporation and discharge of CLA from cell membranes require time. The effects of CLA on cell membranes are assumed to last longer than the ones of other dietary polyunsaturated fatty acids such as omega-3 fatty acids due to their differential positioning at the glycerol back bone of phospholipids (typical cell membrane lipids): polyunsaturated fatty acids are predominantly in the sn-2 position of phospholipids, whereas the *trans*-10, *cis*-12 and *cis*-9, *trans*-11 CLA isomers are mainly in the sn-1 position on the phospholipids. The turnover of fatty acids at sn-1 should be slower than that of sn-2 fatty acids because of the predominant occurrence of phospholipases A2 compared to phospholipases A1. Therefore, the effects of CLA may last for a longer time (Subbaiah et al., 2011).

Even though only low CLA concentrations were reached, significant CLA effects were observed herein. In view of the reduced *PPARγ* expression in the mammary gland of primiparous cows after 42 or 105 days of CLA treatment, the udder as main target tissue

was confirmed. Moreover, the effect on *PPARγ* is a novel observation that adds on to our understanding of the mode of action CLA exert in this particular tissue. In addition, the alterations observed in terms of insulin sensitivity indicate that there are indeed long term systemic effects of the supplementation. The data base is still not solid enough for a final appraisal of CLA supplementation with regard to animal health, but the results elaborated herein, indicate that the meaning of the (subtle) decrease in insulin sensitivity requires further consideration.

From the current results, several differences between the different fat depots studied were observed. First, s.c. and v.c. fat depots were not consistently different when taking the respective v.c. and s.c. fat depots together. Second, there were partly differences between the v.c. fat depots that might partly be explained by the different blood supply and drain. This is of general basic interest, however, the third finding was particularly interesting from a more research applied point of view: the results about differences between different s.c. fat depots were surprising and were observed not only at the molecular (mRNA) level but also on the gross tissue level: in primiparous cows 105 days of CLA treatment resulted in decreasing back fat thickness, but not in decreasing BCS that is scored at additional different locations (von Soosten et al., 2011). Another example was the reduction in *SAA3* and haptoglobin mRNA that was exclusively observed in the s.c. fat depot above the withers but not around the tail head. In conclusion, the common practice of limiting assessments of s.c. fat depots to the tail head location limits the results to this very depot and does not justify extrapolation to the entire s.c. fat depots. Moreover, in view of the relatively low share that s.c. fat depots have in comparison to the v.c. fat depots investigated herein and the relatively higher mobilization from v.c. fat depots, in particular retroperitoneal fat, future studies addressing the metabolic and regulatory role of AT should take at least one of the v.c. fat depots into consideration for sampling. According to a recently published paper, retroperitoneal fat can indeed be sampled via biopsy using a novel technique for surgery (Locher et al., 2011).

Finally, the adequacy of the control fat supplement that was used in the current studies to be able to provide isoenergetic rations in both the CLA and the control group finally requires some discussion: using stearic acid to replace CLA in the fat supplement for the control groups is a common practice in comparable experimental set-ups and is well justified to achieve isoenergetic nutrient supply. However, not only CLA but also stearic acid is biologically active and exerts also regulatory functions, e.g. treating murine

adipocytes *in vitro* with saturated fat vs. unsaturated fat reportedly stimulates haptoglobin (Bueno et al., 2010). Indeed, stearic acid as well as different isomers of linoleic acid were defined as ligand for a specific nutrient sensing receptor, i.e. GPR40 (Briscoe et al., 2003; Briscoe et al., 2006). Therefore, some of the biological activities we attributed to CLA herein might in fact be at least partly due to effects induced in the control group. For example, in a preliminary work, we have shown that GPR40 mRNA was increased in liver and in retroperitoneal and omental fat depots after 105 days of CLA treatment (Saremi et al., 2012). It is not yet known whether activation of this receptor by its ligand will regulate its rate of transcription, but if so, medium and long chain fatty acids have in general to be considered as ligands thus questioning the specificity of a CLA effect in this set-up of control feeding. However, alternative fatty acids for control feed supplements will yield similar problems and a valid solution to the problem is not conceivable.

8. Summary

Dairy cows undergo a time of negative energy balance during the first weeks of lactation and have to mobilize body reserves mainly body fat to cover the needs of milk production. Orchestrated hormonal and metabolic changes support the homeorhetic adaptations whereby the drain of nutrients towards the mammary gland is accomplished by decreasing the sensitivity for insulin in other peripheral organs. However, the role of newly discovered adipokines (i.e. messenger molecules secreted from adipose tissue) and their respective receptors as well as of nuclear receptors as key regulators of gene expression was only scarcely investigated in dairy cows. In particular, studies about the regulatory function of adipose tissue (AT) for metabolism were largely limited to subcutaneous (s.c.) fat depots that is accessible using biopsies. Based on data from monogastric species, differences between s.c. and visceral (v.c.) fat depots are probable, but the respective data in cattle were lacking. The present studies were thus carried out to survey the factors related to insulin sensitivity (IS) in dairy cows; based on the link between local inflammatory reactions and decreased IS in AT, pro-inflammatory cytokines and acute phase proteins were additionally addressed. In addition, potential effects of a dietary supplementation with conjugated linoleic acids (CLA) that knowingly reduces milk fat and might thus alleviate the negative energy balance at the onset of lactation were tested.

In two trials, Holstein cows were allocated to either a CLA group (100 g/d of a fat supplement containing 12% each of the *cis*-9, *trans*-11 and the *trans*-10, *cis*-12 isomer) or a control group (100 g/d of a control fat in which CLA was substituted by stearic acid). In trial 1, liver and s.c. fat depot from tail head as well as blood samples were collected from pluriparous cows from d -21 to d 252 relative to calving whereby the supplements were fed from d 1 to d 182. In trial 2, primiparous cows were accordingly treated and tissue samples were collected at slaughter on days 1, 42 and 105 p.p. Samples were collected from liver, mammary gland, *M. semitendinosus*, pancreas, and 3 different v.c. fat depots (omental, mesenterial, and retroperitoneal) and from 3 s.c. locations (tail head, withers and sternum) of AT.

The main focus of this thesis was on gene expression and therefore appropriate reference genes (REF) had to be established in first place. From 7 REF measured, the most stable ones were identified using geNorm^{PLUS} (**manuscript 1**) for comparisons within tissues and for tissue combinations. By providing both new and stable REF, the current status of

normalization in gene expression analysis in different tissues of dairy cows could thereby be improved. Moreover, REF were suggested for some of the adipose depots for which stable REF have previously not been investigated at all, i.e. for 2 s.c. locations, i.e. above withers and sternum, and in omental fat. Indeed, the results explicitly discourage from using *GAPDH* as the sole REF if its stability is not approved by one of the known algorithms such as geNorm.

Within **manuscript 2**, we aimed to characterize the lactational changes of the major bovine acute phase protein, i.e. haptoglobin (Hp), in serum and its mRNA expression in different tissues of dairy cows considering also potential effects of CLA supplementation. At the day of parturition, Hp concentrations and tissue mRNA expression were increased compared to a.p. values; thereafter they decreased and remained stable until the end of the observation. Based on the presence of Hp-immuno staining in histological sections from different fat depots and Hp mRNA expression in AT and differentiated and undifferentiated adipocytes, Hp was established as an adipokine for the bovine species. In view of the low Hp mRNA abundance in ATs as compared to liver and the correlation between tissue mRNA abundance and Hp serum concentrations that was limited to liver, Hp from AT will not or only marginally contribute to the circulating Hp concentrations. Our data are thus in support of the liver being the main site of Hp expression and secretion. In humans, Hp was suggested as marker of adiposity, but the mRNA abundance values and the lack of correlations between Hp and body weight, body fat mass or body condition score observed herein, are not in support of a relation between serum Hp concentrations and body fat content. The moderate correlation between Hp and NEFA and BHB concentrations in serum underpins the relationship between hepatic lipidosis and Hp secretion. Hp in serum was not affected by supplementation or withdrawal of CLA. Based on the observation that the Hp mRNA abundance in 2 out of 6 different fat depots was reduced in CLA treated primiparous cows as compared to the control group, CLA might exert local anti-inflammatory effects within AT. To address such effects in more detail, future studies might be extended to the level of the protein.

In an analogous study (**manuscript 3**), another major acute phase protein, serum amyloid A (SAA) was investigated in different tissues of dairy cows taking into account potential changes with time of lactation and effects of dietary supplementation with CLA. The focus was on fat depots from different localizations. The pattern of *SAA3* (one of the *SAA* isoforms) mRNA abundance throughout lactation was similar to Hp with maximal

abundances around parturition. We demonstrated the presence of *SAA3* mRNA in different v.c. and s.c. fat depots as well as in cultured bovine preadipocytes and differentiating adipocytes. Moreover, the presence of SAA in AT was confirmed at the level of the protein thus confirming that SAA3 - similar to Hp - is an adipokine in cattle, too. The highest *SAA3* mRNA expression was observed in the mammary gland; AT ranked second whereas liver seems not to be a major site of expression of the *SAA3* isoform. CLA treatment reduced the *SAA3* mRNA abundance in 2 s.c. fat depots and in muscle tissue. Therefore, a similar local anti-inflammatory effect as mentioned for Hp may also exist in case of *SAA3*. As to whether this effect will be seen at the level of plasma or at the level of tissue protein expression remained to be clarified.

In **manuscript 4**, the mRNA expression profiles of genes related to IS were characterized encompassing an entire lactation cycle in pluriparous cows and the first 105 d in milk in primiparous cows. The studied genes include adiponectin (*ADIPOQ)*, leptin (*LEP)*, their receptors (*LEPR*, *LEPRB*, *ADIPOR1*, and *ADIPOR2*), two nuclear receptor isoforms (*PPARγ* and *PPARγ2*) and 2 pro-inflammatory cytokines (*TNF-α* and *IL-6*). To be able to relate these data to whole-body IS, an index (RQUICKI) was calculated from the serum concentrations of glucose, insulin, and NEFA. In pluriparous cows, the mRNA expression of the aforementioned genes was mostly decreased from d -21 a.p. to day 21 p.p. in s.c. fat depot with the exception of *TNF-α* which was stably expressed. In liver, increases were observed for *LEPR*, *LEPRB* and *ADIPOR2*, and decreases for all other hepatic target mRNAs except *TNF-α* and *ADIPOR1* which remained constant in this time. In later lactation, a.p. values were reached again and were largely maintained until d 252 p.p. A reduction in IS was confirmed after parturition using RQUICKI index lasting even after withdrawal of CLA. The groups treated with CLA vs. control fat had lower mRNA expression of *PPARγ* in liver and *PPARγ2* in s.c.AT. In addition, CLA vs. Control group had higher expression of *LEPRB* and *TNF-α* in liver. Cows of the CLA group had also higher insulin concentrations and reduced systemic IS as estimated by the RQUICKI index persisting after the end of CLA supplementation similar to *PPARγ* mRNA in liver. In primiparous cows, changes with the duration of lactation were observed for most of the target mRNAs, except *LEP*, but not in all tissues investigated; time course and direction of change were partly divergent between the different tissues. In both trials, the observed shifts in the mRNA expression of modulators of IS like the *ADIPOQ* and the *LEP* system in different tissues of cows were in accordance to the known reduction in IS during the

transition period and its improvement thereafter. CLA treatment for 105 days decreased the mRNA abundance of *ADIPOQ*, *ADIPOR2*, and *TNF-α* in v.c. fat depots and of *PPARγ2* in the mammary gland. The results of these studies provide expression profiles of genes that are particularly related to the heterogeneous, i.e. depot-specific, regulatory functions of AT in lactating dairy cows. The known effect of CLA inhibiting milk fat synthesis might at least be partly explained by the down-regulation of *PPARγ2* in the mammary gland observed herein. Moreover, the results summarized herein support the notion that CLA treatment indeed decreases IS in dairy cows. The importance of the CLA induced effects on IS for animal health can presently not be finally assessed due to lack of validated reference values for IS in high yielding dairy cows. However, the reduced IS indicated by the RQUICKI estimates needs substantiation by direct assessments e.g. hyperinsulinemic euglycemic clamp. Besides the comprehensive longitudinal characterization of the mRNA expression of genes linked to IS and to inflammatory reactions, the works of this thesis support both local and systemic effects of CLA treatment on IS, and point to rather local than systemic anti-inflammatory effects. These findings should be further substantiated at the level of the protein.

9. Zusammenfassung

Während den ersten Wochen der Laktation befinden sich Milchkühe in einer Phase der negativen Energiebilanz. Der Austrag von Energie über die Milch, der durch die demgegenüber nur langsame Steigerung der Futteraufnahme noch nicht ausgeglichen werden kann, macht die Mobilisierung von Körperreserven, primär von Fett notwendig. Hormonelle und metabolische Veränderungen ermöglichen über eine verringerte IS der peripheren Organe den Fluss der Nährstoffe zur Milchdrüse und unterstützen somit die homöorethische Anpassung. Die Bedeutung von neu entdeckten Adipokinen (vom Fettgewebe sekretierte Botenstoffe), deren entsprechenden Rezeptoren sowie von Kernrezeptoren als Schlüsselregulatoren der Genexpression wurde bei Milchkühen bisher kaum untersucht. Insbesondere Studien zur regulatorischen Funktion des Fettgewebes auf den Stoffwechsel bezogen sich hauptsächlich auf das mittels Biopsie erreichbare subkutane Fettgewebe. Daten basierend auf Untersuchungen von Monogastriern weisen aber auf Unterschiede zwischen subkutanen und viszeralen Fettdepots hin; entsprechende Daten fehlten jedoch weitestgehend für Wiederkäuer und somit auch für das Rind. Ziel der vorliegenden Studien war die Untersuchung von Faktoren, die bei Milchkühen mit IS in Verbindung stehen. Aufgrund der Verbindung zwischen lokalen Entzündungsreaktionen und verminderter Insulinsensitivität (IS) im Fettgewebe wurden auch pro-inflammatorischer Zytokine sowie Akut Phase Proteinen (APP) berücksichtigt. Darüber hinaus wurde der Einfluss konjugierter Linolsäuren (CLA) untersucht, welche den Milchfettgehalt reduzieren und dadurch die negative Energiebilanz zu Beginn der Laktation mindern könnten.

In zwei Versuchen wurden Holstein Kühe in eine CLA-Gruppe (100 g Fettsupplement pro Tag mit je 10% *cis*-9, *trans*-11 und *trans*-10, *cis*-12 Isomer) oder eine Kontrollgruppe (100 g Fettsupplement pro Tag, Ersatz der CLA durch Stearinsäure) eingeteilt. In Versuch 1 erfolgte die Supplementation bei pluriparen Kühen im Zeitraum zwischen Tag 1 und 182 nach der Kalbung; Biopsien von Leber, subkutanem Fett sowie Blutproben wurden zwischen Tag 21 a.p. und Tag 252 p.p. entnommen. In Versuch 2 erfolgte eine entsprechende Fütterung von primiparen Kühen. An Tag 1, 42 und 105 p.p. wurden bei der Schlachtung der Tiere Proben von Leber, Milchdrüse, *M. semitendinosus*, Pankreas, drei verschiedenen subkutanen Fettdepots (an Schwanzansatz, Widerrist und Sternum) sowie von drei viszeralen Depots (omental, mesenterisch und retroperitoneal) genommen.

Der Schwerpunkt dieser Arbeit lag auf der Analyse der Genexpression; aufgrund dessen

erfolgte im ersten Schritt die Etablierung geeigneter Referenzgene (REF). Aus sieben gemessenen REF wurden mittels geNorm^{PLUS} (**Manuskript 1**) die stabilsten Gene ausgewählt und damit Vergleiche innerhalb und zwischen den einzelnen Geweben ermöglicht. Durch die Bereitstellung stabiler REF konnte der gegenwärtige Status der Normalisierung in verschiedenen Geweben von Milchkühen verbessert werden. Darüber hinaus wurden für das subkutane Fettgewebe an Widerrist und Sternum sowie für das omentale Fett gänzlich neue REF eingemessen. Die Ergebnisse weisen darauf hin, dass *GAPDH* nicht als alleiniges REF verwendet werden sollte, da dessen Stabilität nicht mit einem bekannten Algorithmus, wie z.B. geNorm, bestätigt werden konnte.

In **Manuskript 2** erfolgte die Darstellung der laktationsbedingten Veränderungen eines bedeutenden bovinen APP, des Haptoglobins (Hp), im Serum sowie dessen mRNA-Expression in verschiedenen Geweben von Milchkühen unter Berücksichtigung möglicher Effekte einer CLA-Supplementation. An Tag 1 nach der Geburt zeigten sich sowohl erhöhte Hp-Konzentrationen im Serum als auch eine erhöhte mRNA-Expression. Anschließend sanken die Werte wieder auf ein annähernd konstantes Niveau bis zum Ende der Untersuchungsphase. Auf Basis der positiven Hp-Immunfärbung von Adipozyten in histologischen Gewebeschnitten aus verschiedenen Fettdepots, der Hp mRNA-Expression in verschiedenen Fettdepots sowie in differenzierten und undifferenzierten (Prä-) Adipozyten konnte Hp beim Rind als ein Adipokin eingeordnet werden. In Anbetracht der nur geringen Hp mRNA-Expression im Fettgewebe im Vergleich zur Leber und der Korrelation der Hp-Serumkonzentration, die nur für die Hp mRNA-Expression in der Leber, nicht aber in den Fettgeweben bestand, ist die Leber als Hauptort der Hp-Expression und -Sekretion bestätigt. Beim Menschen wurden Hp-Blutwerte als Marker für Adipositas diskutiert. Nach den hier vorgelegten Daten trägt Hp aus dem Fettgewebe beim Rind aber vermutlich nicht oder nur zu einem geringen Anteil zu den zirkulierenden Hp-Konzentrationen bei. Die hier ermittelten mRNA-Verteilungswerte sowie die fehlende Korrelation zwischen Hp und Körpergewicht, Körperfettmasse und BCS lassen bei Milchkühen keine Beziehung zwischen Hp-Serumkonzentrationen und Körperfettgehalt vermuten. Die mittlere Korrelationen zwischen Hp und den NEFA- sowie BHB-Konzentrationen im Serum bestätigen den Zusammenhang zwischen hepatischer Lipidose und Hp-Sekretion. Die Supplementierung mit CLA hatte keinen Einfluss auf die Hp-Serumkonzentrationen. Aufgrund der Beobachtung, dass die Hp mRNA-Expression in zwei der sechs untersuchten Fettdepots in CLA-behandelten, primiparen Kühen im Vergleich zur Kontrollgruppe reduziert war,

könnte CLA lokal anti-inflammatorische Effekte im Fettgewebe ausüben. Um diese Effekte genauer bewerten zu können, sollte in zukünftigen Studien zusätzlich zur mRNA auch das Protein berücksichtigt werden.

In einer analogen Studie (**Manuskript 3**) erfolgte die Untersuchung des APP Serum Amyloid A (*SAA*) in verschiedenen Fettgeweben von Milchkühen unter Berücksichtigung möglicher laktationsbedingter Veränderungen sowie einer CLA-Supplementation. Die Expressionsrate der *SAA3*-mRNA (einer *SAA* Isoform) während der Laktation entsprach der von Hp und zeigte die höchsten Werte um den Zeitraum der Geburt. Zudem konnte die *SAA3*-mRNA in verschiedenen subkutanen und viszeralen Fettdepots sowie in Präadipozyten und differenzierten Adipozyten aus Zellkultur nachgewiesen werden. Diese Ergebnisse konnten auf Proteinebene bestätigt werden und beweisen somit, dass *SAA3* beim Rind ein Adipokin ist. Die höchste *SAA3* mRNA-Expression wurde in der Milchdrüse gefunden, gefolgt vom Fettgewebe. Die Leber scheint hingegen keinen Hauptexpressionsort der *SAA3*-Isoform darzustellen. Die CLA-Behandlung verringerte die *SAA3*- mRNA-Expression in zwei subkutanen Fettgeweben sowie im Muskelgewebe. Somit könnte *SAA3* einen ähnlichen lokalen, anti-inflammatorischen Effekt haben wie Hp. Ob dieser Effekt auch auf systemisch auf Ebene des Proteins existiert, bleibt zu klären.

In **Manuskript 4** erfolgte die Charakterisierung der mRNA-Expressionsprofile von Genen, die im Zusammenhang mit der IS stehen. Die Untersuchungen umfassten einen vollständigen Laktationszyklus bei pluriparen Milchkühen sowie die ersten 105 Laktationstage bei primiparen Kühen. Die mRNA folgender Gene wurde quantifiziert: Adiponektin (*ADIPOQ*), Leptin (*LEP*), deren Rezeptoren (*LEPR*, *LEPRB*, *ADIPOR1*, *ADIPOR2*), zwei Kernrezeptorisoformen (*PPARγ* und *PPARγ2*), sowie zwei pro-inflammatorische Zytokine (*TNF-α* und *IL-6*). Um diese Daten mit der IS in Beziehung setzen zu können, erfolgte die Berechnung eines Indexes (RQUICKI) aus den Serumkonzentrationen von Glucose, Insulin und NEFA. Bei pluriparen Kühen zeigten die zuvor genannten Gene im subkutanen Fettgewebe eine verringerte mRNA-Expression zwischen Tag 21 a.p. und 21 Tage p.p. Einzige Ausnahme stellte *TNF-α* dar, dessen mRNA unverändert blieb. In der Leber konnten Erhöhungen für LEPR, *LEPRB* und *ADIPOQ* sowie Verringerungen für die übrigen hepatischen Zielgene mit Ausnahme von *TNF-α* und *ADIPOR1* nachgewiesen werden. *TNF-α* und *ADIPOR1* zeigten ein konstantes Expressionsniveau. In der späten Laktation erreichten die Werte wieder das

vorgeburtliche Niveau und verblieben weitgehend konstant bis Tag 252 p.p. Nach der Geburt zeigte sich anhand des RQUICKI eine Reduktion der IS, die über den Zeitraum der CLA-Supplementation hinausging. Die CLA-Gruppen wiesen im Vergleich zu den Kontrollgruppen eine niedrigere mRNA-Expression von *PPARγ* in der Leber und *PPARγ2* im subkutanen Fettgewebe auf, zudem verfügten sie über eine höhere Expressionsrate von *LEPRB* und *TNF-α* in der Leber. Die Tiere der CLA-Gruppe zeigten, gemessen am RQUICKI-Index, des weiteren höhere Insulinkonzentrationen sowie eine reduzierte systemische IS, die über das Ende der CLA-Supplementation hinausging. Bei den primiparen Tieren unterlagen die meisten Parameter zeitlichen Veränderungen, jedoch nicht in allen Geweben und mit Ausnahme von *LEP*. Der zeitliche Verlauf sowie die Änderungsrichtung stellten sich teilweise gegenläufig zwischen den Geweben dar. In beiden Versuchen entsprachen die Änderungen der mRNA-Expressionen von Modulatoren der IS wie *ADIPOQ* und dem *LEP*-System der Reduktion der IS während der Transitionsperiode und der Verbesserung danach. Die CLA-Supplementation über einen Zeitraum von 105 Tagen verringerte die mRNA-Expression von *ADIPOQ*, *ADIPOR2* und *TNF-α* im viszeralen Fettgewebe und von *PPARγ2* in der Milchdrüse. Die Ergebnisse dieser Studie stellen Expressionsprofile von Genen zur Verfügung, die sich auf die depot-spezifische regulatorische Funktion des Fettgewebes bei laktierenden Milchkühen beziehen. Der hemmende Effekt von CLA auf die Milchfettsynthese könnte zumindest teilweise durch die Down-Regulation von *PPARγ2* in der Milchdrüse erklärt werden. Darüber hinaus unterstützen die hier zusammengefassten Ergebnisse die These, dass eine Behandlung mit CLA die IS bei Milchkühen verringert. Die Bedeutung dieses CLA-induzierten Effektes in Hinblick auf die Tiergesundheit kann derzeit aufgrund fehlender Referenzwerte für die IS bei hochleistenden Milchkühen jedoch noch nicht endgültig bewertet werden. Die durch den RQUICKI berechnete reduzierte IS benötigt zudem eine Verifizierung durch eine direkte Messung, wie z.B. einen hyperinsulinämischen euglycämischen Clamp. Neben der umfassenden longitudinalen Charakterisierung der mRNA-Expression von Genen, die in Zusammenhang mit IS und Entzündungsreaktionen stehen, weist diese Arbeit sowohl auf lokale als auch auf systemische Effekte einer CLA-Behandlung in Bezug auf die IS hin. Des Weiteren deuten die Ergebnisse eher auf lokale als auf systemische anti-inflammatorische Effekte. Zur Bestätigung sollten diese Ergebnisse auf die Proteinebene erweitert werden.

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12. Publications derived from this doctorate thesis and from related works

12.1. Papers and manuscripts

1) **Saremi, B.,** Al-Dawood, A., Winand, S., Müller, U., Pappritz, J., von Soosten, D., Rehage, J., Dänicke, S., Häussler, S., Mielenz, M. and H. Sauerwein. 2012. Characterization of the serum concentrations and tissue expression of haptoglobin focusing on its potential relevance as adipokine in dairy cows supplemented with or without conjugated linoleic acids throughout lactation. Vet. Immunol. Immunopathol. 146 (3-4): 201-211 (Link-PDF).
2) **Saremi, B.,** Sauerwein, H., Dänicke, S. and M. Mielenz. 2012. *Technical note:* Identification of reference genes for gene expression studies in different bovine tissues focusing on different fat depots. J. Dairy Sci. 95:3131-3138 (Link-PDF).
3) Sauerwein, H., **Saremi, B.,** Pappritz J., von Soosten, D., Meyer, U., Dänicke, S., and M. Mielenz. 2013. *Short Communication*: Aquaporin-7 mRNA in subcutaneous and visceral adipose tissues of dairy cows: long term physiological and conjugated linoleic acids (CLA) induced changes. J. Dairy Sci. doi: 10.3168/jds.2012-6363).
4) **Saremi, B.,** Mielenz, M., Rahman, M.M., Hosseini, A., Kopp, C, Dänicke, S. and H. Sauerwein. Hepatic and extrahepatic expression of serum amyloid A3 during lactation in dairy cows treated with or without conjugated linoleic acids (Submitted, under revision).
5) **Saremi, B.,** Winand, S., Friedrichs, P., Kinoshita, A., Rehage, J., Dänicke, S., Häussler, S., Breves, G., Mielenz M. and H. Sauerwein. Physiological and conjugated linoleic acids induced changes in the expression of genes related to insulin sensitivity and fat metabolism in different tissues of dairy cows during lactation (Submitted, under revision).
6) **Saremi, B.,** Dänicke, S., Mielenz, M. and H. Sauerwein. Increased pigment epithelium-derived factor expression in adipose tissues associated with lactation-induced body weight loss in non-obese pluriparous cows is suppressed by conjugated linoleic acids (in preparation).

12.2. ABSTRACTS IN CONFERENCES (limited to first authorship presentations)

1) **Saremi, B.,** Winand, S., Friedrichs, P., Sauerwein, H., Dänicke, S. and M. Mielenz. 2012. Adiponectin and leptin system: long term physiological and conjugated linoleic acid induced changes (*oral presentation*). Book of Abstracts of the 63st Annual Meeting of the European Federation of Animal Science, 27.08.-31.08.2012,

Bratislava, Slovakia, Page 64, ISBN 978-90-8686-206-1. (link-PDF) **(Winner of the Best Paper Award)**

2) **Saremi, B.,** Sauerwein, H., von Soosten, D., Dänicke, S. and M. Mielenz. 2012. Tissue dependent expression of G-protein couple receptor (GPR) 40, 41, 43, 109A mRNA in early lactation dairy cows treated with conjugated linoleic acids (CLA) and long chain fatty acids (LCFA) (*oral presentation*). J. Dairy Sci. Vol. 95 (E-Suppl. 2): 703-704 (link-PDF).
3) **Saremi, B.,** Winand, S., Dänicke, S., Pappritz, J., von Soosten, D., Sauerwein, H. and M. Mielenz. 2012. mRNA expression of a novel adipokine - Pigment epithelium-derived factor (PEDF) - in various tissues from dairy cows receiving supplements with or without conjugated linoleic acids (CLA) *(oral presentation).* J. Dairy Sci. Vol. 95 (E-Suppl. 2): 703 (linkPDF).
4) **Saremi, B.,** Winand, S., Pappritz, J., Dänicke, S., Mielenz, M., Rahman, M.M. and H. Sauerwein. 2012. Serum amyloid A3 (SAA3) mRNA in liver and adipose tissue of dairy cows supplemented with or without conjugated linoleic acids (CLA): a whole lactation cycle study *(oral presentation).* J. Dairy Sci. Vol. 95 (E-Suppl. 2): 705-706 (link-PDF).
5) **Saremi, B.,** Sauerwein, H., von Soosten, D., Dänicke, S. and M. Mielenz, 2012. Tumor necrosis factor-α (TNF-α) mRNA expression in early lactation in different tissues of dairy cows with a focus on different fat depots (*oral presentation*). J. Dairy Sci. Vol. 95 (E-Suppl. 2): 706 (link-PDF).
6) **Saremi, B.,** Sauerwein, H., von Soosten, D., Dänicke, S. and M. Mielenz. 2012. Peroxisome proliferator-activated receptor *gamma*2 (*PPAR*γ2) mRNA is related to body fat content of dairy cows and is decreased by CLA treatment in the mammary gland (*poster*). Proc. Soc. Nutr. Physiol. 21.
7) **Saremi, B.,** Sauerwein, H., von Soosten, D., Dänicke, S., Akter, S.H., Häussler, S. and M. Mielenz. 2011. Conjugated linoleic acids (CLA) and lactation related changes of leptin and the long form leptin receptor (ObRb) mRNA abundance in different bovine tissues and their correlation to individual fat depot mass and adipocyte cell size (*poster*). Book of Abstracts of the 7th International Conference on Farm Animal Endocrinology, 24.08.-26.08.2011, Bern, Switzerland. Page 73-74, ISBN 978-3-033-03089-3.
8) **Saremi, B.,** Sauerwein, H., von Soosten, D., Dänicke, S. and M. Mielenz. 2011. Adiponectin system and peroxisome proliferator-activated receptor *gamma* (*PPAR*γ)

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9) **Saremi, B.,** Mielenz, M., von Soosten, D., Dänicke, S. and H. Sauerwein. 2011. Conjugated linoleic acids (CLA) and lactation related changes of serum amyloid A3 (SAA3) and IL-6 mRNA abundance in different bovine tissues with a focus on different adipose depots (*poster*). J. Dairy Sci. Vol. 94 (E-Suppl. 1): 596 (link-PDF).
10) **Saremi, B.,** Mielenz, M., von Soosten, D., Dänicke, S. and H. Sauerwein. 2011. Haptoglobin mRNA abundance in varying localizations of fat tissue and liver of dairy cows fed with or without conjugated linoleic acids during 105 days postpartum (*poster*). Oskar Kellner Symposium: Metabolic Flexibility in Animal and Human Nutrition, 09.09.-11.09.2011, Warnemünde, Germany. Page 53, ISSN 0946-1981.
11) **Saremi, B.,** Mielenz, M., Vorspohl, S., Dänicke, S., Pappritz, J. and H. Sauerwein. 2010. Haptoglobin mRNA expression in bovine adipose and liver tissue: Physiological and conjugated linoleic acids (CLA)-induced changes throughout lactation (*poster*). Book of Abstracts of the 61st Annual Meeting of the European Federation of Animal Science, 23.08.-27.08.2010, Heraklion, Greece, Page 12, ISBN 978-90-8686-152-1 (link-PDF).
12) **Saremi, B.,** Mielenz, M., Hosseini, A., Behrendt, C. and H. Sauerwein. 2010. Haptoglobin expression in different adipose tissues and adipocytes (*in vitro*) of dairy cattle (*poster*). DVG. 19. Symposium der Fachgruppe Physiologie und Biochemie der Deutschen Veterinärmedizinischen Gesellschaft, 14.02.-16.02.2010, Hannover, P25, ISBN 978-3-941703-551.