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Metabolic responses of adipose tissue in the periparturient dairy cow

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List of Abbreviations

ATGL	Adipose triglyceride lipase
BCS	Body condition score
BHBA	Beta-hydroxybutyrate
BSA	Bovine serum albumin
BW	Body weight
cAMP	Cyclic adenosine monophosphate
d	Day
DMEM	Dulbecco's Modified Eagle's Medium
DMI	Dry matter intake
FAS	Fatty acid synthase
GPR109A	G protein-coupled receptor 109A
HC	High concentrate
HRP	Horseradish peroxidase
HSL	Hormone-sensitive lipase
LC	Low concentrate
NAC	Nicotinic acid
NAD(P)	Nicotinamide adenine dinucleotide (phosphate)
NAM	Nicotinamide
NEFA	Non-esterified fatty acid
RPAT	Retroperitoneal adipose tissue
SCAT	Subcutaneous adipose tissue
TG	Triglyceride
TMR	Total mixed ration

1. Summary

“Metabolic responses of adipose tissue in the periparturient dairy cow”
By Dr. Ákos Kenéz

Dairy cows are in a tensed metabolic situation in the time around calving. With the onset of lactation, energy demand increases enormously, which requires mobilization of energy storages, mainly fat reserves. This necessarily involves extensive adaptation processes in adipose tissue depots, the main sites of energy storage in the body. Adipose metabolism has to undergo a switch from an anabolic condition, which is present in the period before calving, into a catabolic condition, which emerges at the time of calving. This is part of the complex metabolic challenge, which high-yielding dairy cows have to face in the early postpartum period, making the control mechanism of lipid mobilization from adipose tissues a critical process in terms of maintaining metabolic health.

For this reason, adipose tissue metabolism has been extensively studied during the recent decades, and several regulatory mechanisms have been identified within the range of physiological and also pathophysiological processes. However, interrelationships between different regulatory mechanisms, as well as the reasons for a huge individual variation in the extent and timing of lipid mobilization are still not yet fully understood. Studying changes in adipose tissue metabolism at different levels would provide a deeper insight into the complex system of metabolic control in the context of homeorhetic adaptation. Therefore, the present study aimed to investigate changes in (1) adipose tissue morphology, (2) molecular mechanisms to activate lipolysis and lipogenesis at a protein level, (3) functional response to adrenergic stimulations, and (4) molecular mechanisms to promote antilipolysis during the periparturient period.

This PhD research was conducted on 20 healthy, multiparous German Holstein cows, which were selected to be homogenous in their body condition, and were kept at the Institute of Animal Nutrition, Federal Research Institute for Animal Health (Braunschweig, Germany). The experimental period started when cows reached the 42nd day before the expected time of parturition and ended on the 100th day of lactation. Cows were fed a diet with two different concentrate-to-roughage ratios, in order to investigate the effect of a high and a low concentrate proportion prepartum (thereby the effect of a higher or lower dietary energy intake). Furthermore, cows were supplemented with dietary nicotinic acid. These dietary treatments were applied to investigate their effect on the extent of lipid mobilization. Adipose

tissue biopsy samples were collected from all cows 42 days prepartum, and 1, 21, and 100 days postpartum. Both subcutaneous and retroperitoneal adipose depots were sampled to study depot-specific differences. These tissues were used to monitor time-related changes in (1) adipocyte size and adipose tissue composition, (2) expression and phosphorylation of key proteins of lipolytic and lipogenic pathways, (3) β-adrenergic functional response in an in vitro lipolysis assay, and (4) antilipolysis induced by nicotinic acid.

Cows included in the study underwent the well-known adaptive processes of the transition period, including increasing milk production coupled with a decreased feed intake, resulting in a negative energy balance, which induced lipid mobilization reflected by increased plasma non-esterified fatty acid concentrations. Nevertheless, dietary interventions (i.e. different concentrate proportion and nicotinic acid supplementation) did not have a significant effect on these variables. Lipid mobilization was also reflected by a decreasing adipose cell size, upregulation of the cellular lipolytic pathway (increased phosphorylation of hormone-sensitive lipase and perilipin), downregulation of lipogenesis (decreased protein expression of fatty acid synthase), but contradictorily, was not associated with an increased in vitro β-adrenergic lipolytic response during early lactation. The antilipolytic effect of nicotinic acid was demonstrated in vitro at molecular and functional levels. In most aspects the two investigated depots, subcutaneous and retroperitoneal adipose tissues did not significantly differ.

The lack of dietary effects was in contrast to the expectations. A large difference in prepartum energy intake was not sufficient to provoke differences in postpartum lipid mobilization, which suggests that other factors, such as genetically determined metabolic capabilities, eventually associated with high or low body condition, might have a superior impact. The antilipolytic effect of nicotinic acid mediated by the G protein-coupled receptor 109A was confirmed only in vitro. The lack of dietary effect may indicate an extensive hepatic bioconversion, preventing nicotinic acid from reaching the adipose tissues in sufficiently high amounts. Morphological and molecular changes were in accordance with a great demand for lipid mobilization to supply energy for milk production and maintenance. Postpartum, the unexpectedly low response of adipose tissues to β-adrenergic stimulation might be explained by changes of β-adrenergic receptor expression; however, it could also indicate the significance of further control mechanisms in triggering an increased lipolysis. To summarize, findings of the current research provide a better understanding of physiological control mechanisms determining adaptation processes in adipose tissues in the periparturient period, by linking morphological, molecular and functional levels of regulation.

2. Zusammenfassung

“Metabolische Antworten des Fettgewebes in der peripartalen Milchkuh”
Von Dr. Ákos Kenéz

Milchkühe befinden sich in der Zeit um die Kalbung in einer angespannten Stoffwechsellage. Mit dem Laktationsbeginn steigt der Energiebedarf enorm an, was die Mobilisierung der Energiespeicher, hauptsächlich Fettreserven nach sich zieht. Dieser Vorgang umfasst unerlässlich eine umfangreiche Adaptation der Fettdepots, welche als wichtigste Stellen der Energiespeicherung dienen. Gleichzeitig mit der Kalbung muss nämlich der Stoffwechsel in den Fettgeweben von Anabolismus, was vor der Kalbung maßgebend ist, auf Katabolismus umgeschaltet werden. Das ist Teil der komplexen metabolischen Herausforderung, die Hochleistungskühe in der frühen postpartalen Periode bewältigen müssen, was die kritische Rolle von Kontrollmechanismen in der Lipidmobilisierung hinsichtlich Erhaltung der metabolischen Gesundheit hervorhebt.

Aus diesem Grund wurde der Stoffwechsel des Fettgewebes im Laufe der letzten Jahrzehnte ausführlich untersucht, und mehrere regulatorische Mechanismen im Rahmen der physiologischen und auch pathophysiologischen Prozesse wurden identifiziert. Dennoch sind Wechselbeziehungen zwischen verschiedenen Regulationsmechanismen, bzw. Ursachen der hohen individuellen Variation im Umfang und Zeitablauf der Lipidmobilisierung nicht vollständig bekannt. Die gleichzeitige Untersuchung der Stoffwechselveränderungen auf mehreren regulatorischen Ebenen würde weitere Erkenntnisse über die komplexe homeorhetische Adaptation liefern. Darum hatte diese Studie das Ziel, Änderungen in (1) Morphologie des Fettgewebes, (2) molekularen Mechanismen für die Aktivierung von Lipolyse und Lipogenese auf Proteinebene, (3) funktioneller Antwort von Geweben auf adrenerge Stimuli, und (4) molekularen Mechanismen für Antilipolyse während der peripartalen Periode zu erforschen.

Diese Studie wurde an 20 gesunden, multiparen Deutschen Holstein Kühen, die homogen in ihrer Körperkondition waren, am Institut für Tierernährung des Friedrich-Loeffler-Instituts durchgeführt. Der Versuch begann, als die Kühe den 42. Tag vor dem errechneten Abkalbetermin erreicht haben, und endete am 100. Laktationstag. Die Diät der Kühe war unterschiedlich in ihrem Kraftfutter-Raufutter Verhältnis, um den Effekt eines niedrigen und eines hohen antepartalen Kraftfutteranteils (und demzufolge einer niedrigen oder hohen Energieaufnahme) zu überprüfen. Zusätzlich wurde der Effekt von diätetisch

zugeführter Nikotinsäure getestet. Das Ziel dieser diätetischen Maßnahmen war, deren Effekt auf den Umfang der Lipidmobilisierung zu untersuchen. Biopsieproben aus den Fettgeweben wurden von allen Kühen am Tag 42 antepartum und an den Tagen 1, 21 und 100 postpartum entnommen. Um depot-spezifische Unterschiede untersuchen zu können, wurden sowohl subkutane als auch retroperitoneale Fettgewebe gewonnen. Diese Gewebeproben wurden für Untersuchungen (1) der Fettzellgröße und Fettgewebezusammensetzung, (2) der Expression und Phosphorylierung von Proteinen mit zentraler Bedeutung für lipolytische und lipogenetische Stoffwechselwege, (3) der β-adrenergen funktionellen Antwort von Geweben in einem in vitro Lipolyse Assay, und (4) der nikotinsäurevermittelten Antilipolyse verwendet.

Die Kühe in dieser Studie haben die allgemein bekannten Adaptationsvorgänge der Transitperiode aufgewiesen: steigende Milchproduktion gekoppelt mit eingeschränkter Futteraufnahme, bzw. eine Lipidmobilisierung ausgelöst von negativer Energiebilanz, die durch erhöhte Plasmakonzentration nichtveresterter Fettsäuren widergespiegelt wurde. Diese Vorgänge wurden von den diätetischen Behandlungen (unterschiedlicher Kraftfutteranteil und Nikotinsäuresupplementation) nicht signifikant beeinflusst. Die Lipidmobilisierung in der Frühlaktation war auch mit einer Verkleinerung der Fettzellgröße, einer Hochregulation des zellulären lipolytischen Stoffwechselweges (erhöhte Phosphorylierung der hormonsensitiven Lipase und Perilipin) und einer Herunterregulation der Lipogenese (verminderte Proteinexpression der Fettsäuresynthase) assoziiert, aber nicht mit einer Steigerung der in vitro β-adrenergen lipolytischen Antwort. Der antilipolytische Effekt von Nikotinsäure konnte in vitro auf molekularer und funktioneller Ebene gezeigt werden. Die zwei untersuchten Fettdepots, subkutanes und retroperitoneales Fett haben sich in den meisten Parameter nicht signifikant unterschieden.

Der mangelnde diätetische Einfluss war gegensätzlich zur initialen Hypothese. Ein bedeutsamer Unterschied in der antepartalen Energieaufnahme hat nicht ausgereicht, um Unterschiede in der postpartalen Lipidmobilisierung zu induzieren, was impliziert, dass andere Einflussfaktoren, wie genetisch bestimmte metabolische Kapazitäten, eventuell assoziiert mit niedriger oder höher Körperkondition, eine übergeordnete Rolle spielen können. Der antilipolytische Effekt von Nikotinsäure, vermittelt durch den *G protein-coupled receptor 109A* konnte nur in vitro bestätigt werden. Der mangelnde diätetische Effekt kann durch hepatische Biokonversation bedingt sein, was eine ausreichend hohe Konzentration an Nikotinsäure in den Fettgeweben verhindern kann. Die morphologischen und molekularen Änderungen waren in Übereinstimmung mit dem enormen Bedarf an Lipidmobilisierung um

Energie für Milchproduktion und Erhaltung zu sichern. Die unerwartete verminderte Antwort der Fettgewebe auf β-adrenerge Stimulation postpartum kann einerseits mit Änderungen in der Expression der β-Rezeptoren erklärt werden, andererseits kann dieses auf die Wichtigkeit von weiteren Kontrollmechanismen für die Steigerung der Lipolyse hinweisen. Zusammenfassend tragen die Befunde dieser Studie durch Verknüpfung von morphologischen, molekularen und funktionellen Aspekten dazu bei, die physiologischen Regulationsmechanismen, die die peripartale Adaptationsmechanismen in den Fettgeweben bestimmen besser zu verstehen.

3. Introduction

3.1. Metabolic Status of Dairy Cows in the Periparturient Period

Dairy cows have a tensed metabolic situation in the period around calving. Energy intake is typically reduced because of a decreased dry matter intake, and energy demand is highly increased because of the onset of milk production. This triggers a negative energy balance which affects high-yielding dairy cows between 2 weeks prepartum and 10 weeks postpartum (Drackley, 1999; Loor et al., 2013; Drackley et al., 2005). To compensate for the negative energy balance, body reserves are mobilized (about 0.6 kg/day fat and 0.04 kg/day protein within the first 8 weeks after calving; Tamminga et al., 1997). This mobilization is part of an orchestrated network of adaptation processes between metabolic pathways affecting several organs including but not limited to adipose tissues, liver, muscles and mammary gland (Vernon, 2005; Roche et al., 2013). Furthermore, the aim of these adaptive changes is to facilitate nutrient partitioning towards the mammary gland, as genetic merit of high-yielding dairy cows determine milk production to have the highest priority, even at the cost of body reserves (Bauman and Bruce Currie, 1980; Ingvartsen, 2006).

The need for mobilization of fat reserves substantially affects the metabolism of adipose tissue being the main organ for energy storage. This includes the breakdown of triglycerides **(TG)** stored in adipocytes and the release of non-esterified fatty acids **(NEFA)** and glycerol (McNamara, 1994; Tamminga et al., 1997; Koltes and Spurlock, 2011). The increased amount of NEFA released to the blood is taken up primarily by the liver where it becomes fully oxidized under physiological conditions to gain energy. However, if the amount of NEFA arriving in the liver is too high or if the oxidative capacity of the liver is exceeded (or both), then instead of a total oxidation, NEFAs are partially metabolized to form ketone bodies or esterified to form TGs. Accordingly, elevated plasma NEFA concentrations can typically be detected between 1 week prepartum and 2 weeks postpartum, and elevated plasma beta-hydroxybutyrate **(BHBA)** concentrations as well as increased liver TG content during the subsequent weeks (Drackley, 1999; Vernon, 2005; Drackley et al., 2005). Postpartum lipid mobilization is also reflected by a decrease of body condition score **(BCS)**, body weight and back fat thickness (Reid et al., 1986; Pires et al., 2013).

As a result of the above-mentioned metabolic events, the early postpartum period is of critical importance in terms of metabolic health (Goff and Horst, 1997; Loor et al., 2013). This time is often affected by pathophysiological events, which can be attributed, at least in

part, to an insufficient capacity of the cow to cope with the complex metabolic challenge represented by the intensive lipid mobilization (Roche et al., 2013). The discrepancy between mobilization and oxidative capacity leads to metabolic imbalance, which affects many of the high-yielding cows. Typical signs of metabolic dysregulation involve hyperketonemia and hepatic steatosis, which often end up in clinical diseases such as ketoacidosis, reproductive disorders, displaced abomasum, etc. (as reviewed in: Drackley, 1999; Drackley et al., 2005; Ingvartsen, 2006). In turn, these have the consequence of a reduced animal health and welfare, and increased economic losses (Loor et al., 2013). Besides other measures in dairy management, feeding strategies have been developed and are constantly improved to handle and most importantly, to avoid metabolic disturbances associated with early lactation in dairy herds (as reviewed in: Overton and Waldron, 2004; Ingvartsen, 2006; Roche et al., 2013).

3.2. Adaptation Processes of Adipose Tissues during the Periparturient Period

Adipose tissue metabolism has to be continuously subjected to adjustments driven by the varying energy balance and homeorhetic environment during the lactation cycle. In this context, the main challenge that adipose tissues have to face is the need for overcoming the energy deficiency, which emerges at the onset of lactation, and provokes a strong catabolic condition. On the other hand, late lactation and the dry period are characterized by the dominance of an anabolic status allowing storage of TG in the adipose tissues (Drackley, 1999; Ingvartsen, 2006). Therefore, a cyclicity of catabolic and anabolic conditions can be observed in adipose tissue metabolism, which repeats itself with each lactation cycle. Accordingly, lipolysis is upregulated and lipogenesis is downregulated during early lactation, and the other way around when cows are in a positive energy balance (McNamara, 1994, 2012; Rocco and McNamara, 2013).

The mobilization of adipose reserves during early lactation occurs under neuroendocrine regulation including the adrenergic stimulation of the β-adrenoceptor – adenylate cyclase – protein kinase A – hormone-sensitive lipase axis which is the canonical enzymatic pathway of lipolysis as shown in **Fig. 1** (McNamara, 1991; Holm et al., 2000). At the same time insulin sensitivity, representing the main lipogenic and antilipolytic source of regulation, is decreased (De Koster and Opsomer, 2013). Activation of the cellular lipolytic pathway in bovine adipose tissues could also be reached in vitro by stimulation with adrenergic agonists such as norepinephrine which stimulates both α- and β-receptors, or isoproterenol which is selective for the β-receptors (Smith and McNamara, 1989; Khan et al.,

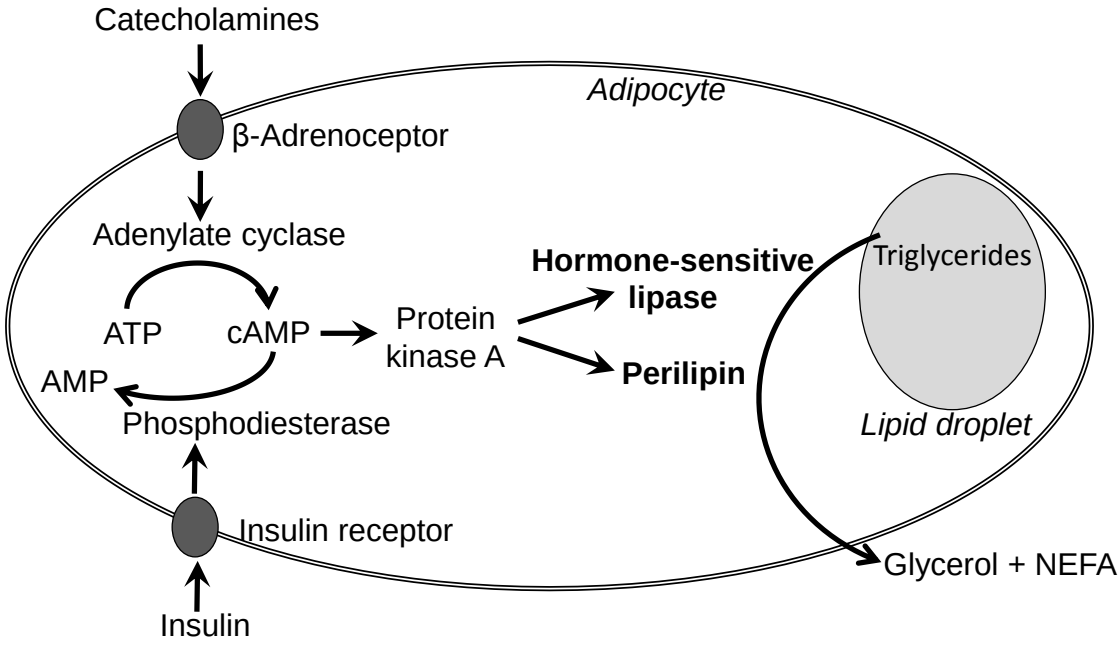

Figure 1. Cellular pathways to control lipolysis. Catecholamines such as epinephrine and isoproterenol promote activation of lipolytic proteins such as hormone-sensitive lipase and perilipin by phosphorylation. This induces triglyceride hydrolysis. Insulin antagonizes lipolysis.

2013). During this in vitro stimulation, the released amount of glycerol and NEFA, i.e. the products of TG hydrolysis is considered as a measure of lipolytic or adrenergic response. This response has been shown to vary throughout the periparturient period (Smith and McNamara, 1989; Khan et al., 2013). The β-adrenergic response of adipose tissues is certainly modulated by the expression of β-adrenergic receptors. Earlier studies showed that β-adrenergic receptor binding capacity increased during lactation (Jaster and Wegner, 1981), and also the mRNA for β_2-adrenergic receptors was shown to increase postpartum in direct relation to rates of milk production (Sumner and McNamara, 2007). However, the β-adrenergic receptor effect is further modulated at the level of posttranslational modification, i.e. by protein phosphorylation. The phosphorylation of the β-adrenergic receptor was shown to have an inhibitory effect on the receptor action. This is a form of agonist-mediated receptor desensitization, as the activated lipolytic cascade was shown to exert a negative feedback effect on the β-receptors (Hausdorff et al., 1990; Lefkowitz et al., 1990). Furthermore, the activation of lipolysis in bovine adipose tissues was shown to involve the phosphorylation of hormone-sensitive lipase **(HSL)** and perilipin as key molecular targets (Elkins and Spurlock, 2009; Koltes and Spurlock, 2011; Locher et al., 2011; Rocco and McNamara, 2013). The function of these lipid droplet-associated proteins is known to be crucial in TG hydrolysis, as the association between adrenergic stimulation of the tissue and the release of glycerol and

NEFA was demonstrated in vitro using bovine adipose tissue explants (Smith and McNamara, 1989; Rukkwamsuk et al., 1998; Sumner and McNamara, 2007; Khan et al., 2013). Additionally, the direct causal relationship between adrenergic stimulation, increase in HSL phosphorylation and functional lipolytic response was shown as well (Kenéz et al., 2014). Furthermore, reflecting these molecular events associated with the ongoing lipolysis, the decrease of fat depot mass and adipocyte size in the period of early lactation was also described (Akter et al., 2011; von Soosten et al., 2011). Nevertheless, remarkable differences were observed in the extent of these processes between subcutaneous and abdominal adipose tissue depots. These differences were shown both at molecular level, including protein phosphorylation (Locher et al., 2011, 2012), at cellular level, including cell size and rate of apoptosis (Akter et al., 2011; Häussler et al., 2013), and at the level of adipose depot in terms of changes in depot mass (von Soosten et al., 2011). The observed differences especially between subcutaneous adipose tissue **(SCAT)** and retroperitoneal adipose tissue **(RPAT)** suggested a greater metabolic flexibility (i.e. greater capacity for storage and a more dynamical mobilization) for RPAT.

In contrast to lipolysis, which is discussed to be primarily a function of milk energy output, lipogenesis is rather influenced by the actual energy intake. Unlike in monogastric animals, adipose tissues of cows utilize preferentially acetate for fatty acid synthesis. This was shown to be regulated mainly at a transcriptional level (McNamara, 1989; Sumner and McNamara, 2007; Rocco and McNamara, 2013). Fatty acid synthase **(FAS)** is a central enzyme in de novo fatty acid synthesis (Smith, 1994). Besides de novo synthesis, also fatty acids acquired from the blood by lipoprotein lipase activity are esterified in adipocytes. The TGs formed in times of energy surplus are stored in the lipid droplet, the fat-containing compartment of the adipocyte, where they can be mobilized from when energy scarcity emerges (Vernon, 2005; Khan et al., 2013).

This cycle of lipid storage and lipid mobilization necessarily induces extensive adipose tissue remodeling throughout the lactation cycle (Vernon, 1981; Rocco and McNamara, 2013). Persistent lipolysis in adipocytes leads to the reduction of TG storage located in the lipid droplet. Accordingly, in times of a negative energy balance in the postpartum period, the ongoing TG mobilization was shown to be associated with a decreased adipose cell size due to lipid droplet shrinkage (Akter et al., 2011), and in case of total depletion, with a decreased adipose cell number due to apoptotic cell death (Häussler et al., 2013). Later on in the postpartum period when dry matter intake starts to increase, energy balance increases as well and becomes positive again around the 8th week of lactation (however with a large variation in

timing between individuals) (Drackley, 1999; Roche et al., 2013). Consequently, lipogenesis also increases allowing spare energy to get stored in adipose tissues (Vernon, 1981; Khan et al., 2013; Rocco and McNamara, 2013). The latter process has to be associated with cell growth and increasing adipose cell size (hypertrophy), and eventually with preadipocyte differentiation and thereby increased cell number (hyperplasia) as well (Spalding et al., 2008; Hausman et al., 2009; Tchoukalova et al., 2010). The possible ways of adipose tissue remodeling are summarized in **Fig. 2**. These ways are under dynamical adjustments as a function of energy status.

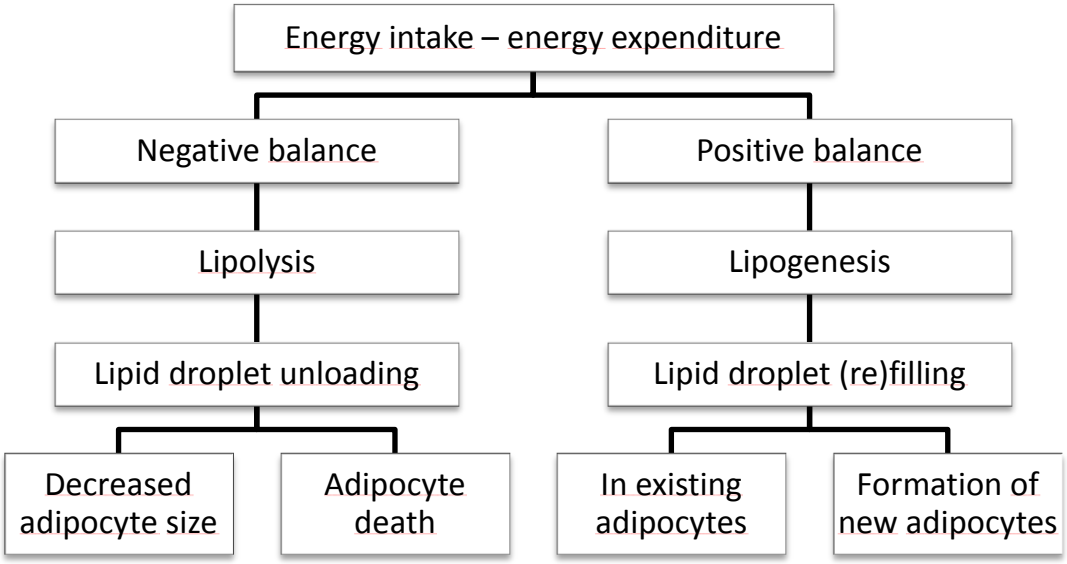

Figure 2. A scheme of adipose tissue remodeling in the context of energy homeostasis and adipocyte turnover. Depending on the actual energy balance, lipolysis and lipogenesis promote changes in triglyceride content of the lipid droplet. Accordingly, lipid mobilization leads to a decreased adipocyte size and eventually to the destruction of the depleted adipocyte. In contrast, triglyceride deposition can cause either hypertrophy of already existing adipocytes or hyperplasia by the differentiation of newly formed adipocytes.

Homeorhetic adaptation processes, including adaptation of adipose tissue metabolism in periparturient cows have been heavily studied during the recent decades, since the integrity of this system is of crucial importance in the context of animal health in dairy farming. As a result, many aspects of physiological regulatory mechanisms within adipose tissues have been revealed, and in several cases the role of these mechanisms in the complex network of physiology, pathophysiology, metabolic imbalance and clinical diseases have been described. Furthermore, a number of metabolic pathways have been shown to be able to be influenced by dietary interventions. However, many “territories” on the “map of metabolic networks” in

adipose tissues are still unrevealed, especially links between different levels of regulation such as between morphological, molecular and functional levels. Furthermore, there are still open questions with regard to how certain dietary factors can affect this metabolic network.

3.3. Dietary Influence on Adipose Tissue Mobilization

As described above, the period around calving (i.e. the transition from pregnancy to lactation) represents a significant metabolic challenge for high-yielding dairy cows. One segment of this complex challenge is to compensate for a negative energy balance by the mobilization of body reserves, and maintaining metabolic health at the same time. In this context, it is desirable to achieve a balanced lipid mobilization to avoid excessive amounts of NEFA overflowing the liver. Therefore, dietary management strategies aiming to restrain lipid mobilization could be beneficial to reduce metabolic stress.

3.3.1. Effect of Dietary Nicotinic Acid Supplementation

Nicotinic acid **(NAC)** or nicotinamide **(NAM)** (commonly referred to as niacin, vitamin B_3) are often supplemented in small amounts to the diet of dairy cows because of their beneficial metabolic effects, even though they can be synthesized also by rumen bacteria and the host itself (Niehoff et al., 2009). Physiologically, they are used to form the coenzymes NAD and NADP, therefore they play a role in a myriad of metabolic pathways. On the other hand, NAC applied in much higher doses was found to exert another physiological effect beyond the commonly known significance as a vitamin: the potential to suppress lipolysis (Tunaru et al., 2003; Pires and Grummer, 2007).

The antilipolytic effect of NAC was initially demonstrated in vitro in rat adipose tissue (Carlson, 1963). Its cellular target, the receptor which is called G protein-coupled receptor 109A **(GPR109A)** in cows (analogous to HM74A in humans and PUMA-G in mice) has been identified as well (Lorenzen et al., 2001; Wise, 2003). The binding of NAC to this receptor was shown to be responsible for its antilipolytic action through inhibiting adenylate cyclase and reducing intracellular levels of cyclic adenosine monophosphate **(cAMP)** in murine adipocytes (Tunaru et al., 2003). Therefore, NAC could presumably counteract the cellular lipolytic pathway (shown in Fig. 1) which is in charge of an enhanced lipid mobilization in periparturient dairy cows.

In feeding trials, dietary NAC supplementation has been shown to affect energy metabolism in dairy cows by lowering plasma NEFA concentrations (Schultz, 1971; Fronk and Schultz, 1979; Dufva et al., 1983; Niehoff et al., 2009). This was confirmed in studies applying rumen-protected NAC as well (Morey et al., 2011; Yuan et al., 2012), after it was shown that only a small proportion of orally administered NAC is bioavailable (Santschi et al., 2005). Reduced plasma NEFA concentrations were observed after post-ruminal infusion of NAC as well (Pires and Grummer, 2007; Pires et al., 2007). Also, the GPR109A protein was shown to be present in bovine adipose tissues (Titgemeyer et al., 2011). Reports regarding the antilipolytic effect of NAM are somewhat inconsistent. In a feeding trial, NAM supplementation of lactating dairy cows was associated with decreased plasma NEFA and BHBA concentrations, indicating a reduced lipid mobilization (Jaster and Ward, 1990). On the other hand, a study investigating receptor binding revealed that NAM is inactive on GPR109A (Lorenzen et al., 2001; Wise, 2003). Nevertheless, the antilipolytic potential of GPR109A agonists on bovine adipose tissues in vitro has not been investigated yet on a protein level.

3.3.2. Effect of Dietary Energy Intake

An optimal body condition at calving is known to be crucial to minimize the risk for postpartum metabolic disorders. The optimal BCS at parturition was suggested to be 3.0-3.25 on a 5-point scale (Roche et al., 2009). A higher body condition was found to be associated with greater rates of lipid mobilization and a greater incidence of peripartal diseases such as ketosis, displaced abomasum, fatty liver and milk fever (as reviewed in: Roche et al., 2009).

For maintaining cows in the optimal BCS range, measures of dietary management such as adjustments of energy intake are suitable. Dietary energy intake was shown to become increased by feeding a diet with a high concentrate proportion (60 % in the total mixed ration **(TMR)** on a dry matter basis) in dairy cows prepartum (Petzold et al., 2014; Schulz et al., 2014). Furthermore, this high concentrate proportion fed prepartum was associated with higher plasma NEFA and BHBA concentrations in the early postpartum period, indicating a greater extent of lipid mobilization (Schulz et al., 2014). The link between prepartum overconditioning and postpartum lipid mobilization has been demonstrated in a number of studies focusing on dairy health in the periparturient period (Garnsworthy and Topps, 1982; Reid et al., 1986; Treacher et al., 1986; Kokkonen et al., 2005; Pires et al., 2013).

3.4. Hypotheses and Aims

Hypothesis #1. Dietary factors can be used to alter periparturient adaptation of adipose tissue metabolism. Lipolysis is known to become upregulated early postpartum; however, this upregulation could be restrained by receptor agonists of the GPR109A, such as NAC. Accordingly, NAC could downregulate lipolysis, leading to a decreased phosphorylation of lipolytic proteins such as HSL and perilipin.

The mechanism for a more extensively increased plasma NEFA concentration in cows with a high BCS could be a greater activation of the cellular lipolytic pathway. Therefore, cows receiving a diet consisting of a high concentrate proportion prepartum, leading to high energy intake, were hypothesized to have a more intensive lipolysis postpartum, reflected by higher protein expression or phosphorylation of HSL and perilipin in their adipose tissues. In contrast, cows with a lower prepartum energy intake were hypothesized to have lower rates of postpartum lipolysis.

Consequently, feeding (1) a diet with NAC supplementation around parturition, or (2) a diet providing a restrained energy intake prepartum would decrease the extent of lipolysis in adipose tissues, and would avoid excessively high plasma NEFA concentrations in transition cows.

Hypothesis #2. Periparturient and dietary-induced changes of metabolism affect morphology, protein expression and phosphorylation, and functional response of subcutaneous and visceral adipose tissues. The transition from anabolism to catabolism requires extensive adipose tissue plasticity, and as such, it has to be reflected by morphological and structural transformation. Therefore, it was expected that changes in histomorphology and tissue composition throughout the periparturient period (i.e. decrease of cell size and TG content, increase of the amount of structural elements) can reflect the remodeling process of adipose tissues.

Moreover, the switch in adipose tissue metabolism in the time around calving has to be reflected at a molecular and functional level as well. In particular, the cellular lipolytic pathway shown in Fig. 1 has to become upregulated during the early postpartum period, which could be achieved by an increased protein expression or protein phosphorylation (activation) of HSL and perilipin. Protein expression of FAS was expected to become downregulated at the same time, in accordance with a lower need for lipogenesis. The upregulation of the lipolytic pathway was hypothesized to be associated with an increased β-

adrenergic responsiveness. By this, the same β-adrenergic signal might trigger a greater lipolytic response in adipose tissues in times of an increased energy demand. With regard to the timing of these changes, it was expected that the upregulation of lipolysis and the increase of β-adrenergic response is already present at the time of calving, in accordance with the appearance of increased plasma NEFA concentrations.

Additionally to the time-dependent changes, it was hypothesized that dietary effects, which modulate the extent of lipolysis, also affect adipose tissue morphology, composition, protein expression and phosphorylation, and β-adrenergic response. Therefore, these parameters were expected to reflect the hypothesized reduced lipolytic effect of dietary NAC supplementation and lower prepartum dietary energy intake.

Aims. This PhD work aimed to assess the effect of the ongoing periparturient period on adipose adaptation processes by investigating metabolic features of adipose tissues of cows at multiple time points, applying both in vitro and in vivo experimental conditions in a basic research approach. Accordingly, adipose tissue biopsy samples were collected from cows at 42 days **(d)** prepartum, and 1 d, 21 d, and 100 d postpartum. The collected tissue samples were aimed to be used for studying changes in (1) morphology, i.e. adipocyte size, and composition, i.e. DNA, TG, β-actin and total protein content, (2) activation of key lipolytic proteins, i.e. expression and phosphorylation of HSL and perilipin, and (3) β-adrenergic response by performing an ex vivo lipolysis assay with isoproterenol stimulation. In order to compare depot-selective features between subcutaneous and visceral adipose depots, both SCAT and RPAT samples were included in these studies.

Additionally to the time-dependent changes in the studied parameters, the effect of the dietary influence was aimed to be evaluated as well, as the collected adipose tissue samples were deriving from cows which were fed a low concentrate **(LC)** or a high concentrate **(HC)** proportion diet with or without NAC supplementation.

The antilipolytic effect of NAC and NAM was aimed to be tested under in vitro and in vivo conditions. In vitro, the effect of NAC and NAM was tested in a lipolysis assay conducted on freshly collected adipose tissue samples, by evaluating HSL phosphorylation and β-adrenergic response. The effect of NAC supplementation in vivo in a feeding trial was tested by monitoring changes in adipose tissue morphology, quantifying the expression and phosphorylation of lipolytic proteins and assessing β-adrenergic response.

The effect of dietary energy intake on the extent of lipid mobilization was aimed to be tested in cows with a LC or HC proportion diet fed prepartum. In particular, the LC diet was

intended to promote less mobilization, due to lower allowance of fat deposition during the dry period, whereas the HC diet was intended to allow more energy getting stored, therefore promoting a more intensive mobilization. The difference in lipid mobilization between the LC and HC groups was expected to be reflected by the size of adipocytes, by the extent of HSL and perilipin phosphorylation, and at a functional level, by the in vitro β-adrenergic response of the adipose tissues.

The novelty of the present work was to investigate morphology, tissue composition, lipolytic protein expression and phosphorylation, and β-adrenergic response all in the same tissue samples, combining in vitro and in vivo approaches and linking different levels of regulation with each other. Furthermore, the changes of these parameters were followed up throughout the periparturient period within the same individual cows. Therefore, the comprehensive interconnection of the obtained data can allow identification of direct causal relationships between distinct elements within the regulatory network of adipose tissue metabolism. To give some examples, this can be achieved e.g. (1) by associating molecular (protein phosphorylation) and functional (β-adrenergic response) patterns identified in an in vitro lipolysis assay, (2) by relating time-dependent changes in activation of lipolytic proteins in vivo (in non-treated tissues) and in vitro (tissues processed in an in vitro lipolysis assay), or (3) by comparing antilipolytic effects of NAC achieved under in vitro (lipolysis assay) and in vivo (feeding trial) conditions. Furthermore, all measurements were conducted both on SCAT and RPAT samples, enabling to compare subcutaneous and visceral adipose tissues to gain more information on depot-selective characteristics of adipose tissue metabolism. Finally, it should be noted that the present study did not investigate metabolic pathways at a gene expression level, but by evaluating protein expression and phosphorylation, which can better predict functional outcome of regulation.

4. Materials and Methods

4.1. Study Design and Experimental Setup

4.1.1. Animals

The investigations of this PhD research were carried out as part of a larger dairy cow research project funded by the German Research Foundation (DFG). The aim of this parent study was to investigate metabolic adaptation processes and pathways, production performance and immune function of periparturient dairy cows under the influence of an energy-dense diet and NAC supplementation. The study was conducted at the Institute of Animal Nutrition, Federal Research Institute for Animal Health (Friedrich-Loeffler-Institute, Braunschweig, Germany), and was approved by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES; Oldenburg, Germany) in agreement with the German Animal Welfare Act (permit number: 33.9-42502-04-11/0444).

For the purpose of the presented PhD research, 20 German Holstein cows were used deriving from the above-mentioned parent study. Cows were in their second, third, or fourth lactation, and were selected for the study to achieve homogeneity in body weight, body condition score, and milk yield of previous lactation, in order to attenuate possible effects of different condition and merit. All animals were kept in a freestall housing system, were clinically healthy, and were dried off 8 weeks before parturition. The experimental period started when cows reached the 42nd d before the expected time of calving, and ended on the 100th d after calving.

4.1.2. Feeding

Diets were formulated according to the recommendations of the Society of Nutrition Physiology (Frankfurt am Main, Germany). Before 42 d prepartum, animals were fed a grass silage-based diet and thereafter they were randomly assigned to one of four dietary treatments arranged in a 2 × 2 factorial design. The experimental factors were LC or HC diet and NAC supplementation (0 or 24 g/d per cow). In the prepartum period, the diet consisted of 30 % grain-based concentrate and 70 % silage-based roughage (on a dry matter basis) or 60 % concentrate and 40 % roughage (on a dry matter basis) for the LC (n = 10) or the HC group (n = 10), respectively. At parturition, concentrate proportion was altered to 30 % in both groups. Postpartum, concentrate proportion was continuously elevated to 50 % within the first 16 or

24 d in the LC or HC group, respectively. Afterwards, concentrate proportion was maintained at 50 % until the end of the study. **Fig. 3** shows an overview of this feeding regimen. Cows were fed ad libitum and had free access to water. Dietary treatments were designed based on previous studies carried out at the same institution, targeting an overfeeding in the dry period to promote adipose tissue anabolism (Petzold et al., 2014; Schulz et al., 2014). Furthermore, NAC was supplemented from 42 d prepartum to 24 d postpartum to the respective groups in form of non-rumen-protected NAC (Mianyang Vanetta Pharmaceutical Technology Co. Ltd., Sichuan, China) mixed into the concentrate feed, at a dose of 24 g/d per cow (cows receiving NAC supplementation: n = 5 in the LC group, and n = 5 in the HC group). This relatively high dose of NAC was chosen based on previous feeding trials studying the antilipolytic potential of NAC using either non-rumen-protected or encapsulated form (Schultz, 1971; Fronk and Schultz, 1979; Dufva et al., 1983; Niehoff et al., 2009; Morey et al., 2011; Yuan et al., 2012). Components and chemical composition of the diet are shown in **Table 1**.

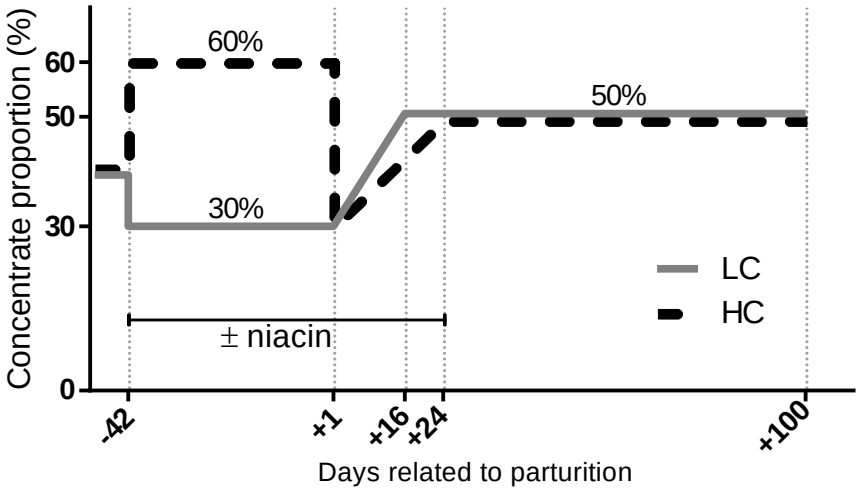

Figure 3. Changes in the concentrate proportion of the diet throughout the experimental period. LC: low concentrate group, HC: high concentrate group.

Dry matter intake and milk yield were registered, and net energy balance (NE_L) was calculated for a larger set of experimental animals included in the parent study, as reported in Tienken et al. (2015, manuscript under revision). Indicative data of these variables were reanalyzed for the 20 cows included in this PhD study are shown in **Fig. 4**.

Table 1. Components and chemical composition of concentrates and roughage. Nicotinic acid was supplemented by providing 1 kg of a concentrate premix containing 24 g nicotinic acid to the corresponding group (NAC-24) each day from 42 d prepartum to 24 d postpartum; the other cows received 1 kg of a control concentrate (NAC-0). Roughage consisted of 50 % corn silage and 50 % grass silage

	Concentrates				***Roughage***	
Item	***Prep.***[1]	***Postp.***[2]	*NAC-0*[3]	*NAC-24*[4]	***Corn silage***	***Grass silage***
Components [%]						
Wheat grain	**49.4**	**49.0**	*50.6*	*49.0*		
Maize	**20.8**	**20.8**	*21.6*	*20.8*		
Soybean meal	**26.8**	**26.8**	*26.8*	*26.8*		
Soybean oil	**1.0**	**1.0**	*1.0*	*1.0*		
Vitamin/mineral premix[5]	**2.0**	**-**	-	-		
Vitamin/mineral premix[6]	**-**	**1.2**	-	-		
Calcium carbonate	**-**	**1.2**	-	-		
Nicotinic acid supplement	-	-	-	*2.4*		
Dry matter [g/kg]	**880**	**873**	*891*	*884*	**353**	**387**
Nutrients [g/kg DM[7]]						
Crude ash	**42**	**47**	*31*	*29*	**36**	**94**
Crude protein	**230**	**228**	*235*	*246*	**120**	**136**
Ether extract	**39**	**39**	*38*	*37*	**29**	**32**
Acid detergent fiber	**45**	**44**	*46*	*50*	**222**	**310**
Neutral detergent fiber	**163**	**152**	*159*	*159*	**461**	**506**
Energy [MJ NEL/kg DM[7]]	**8.5**	**8.4**	*8.6*	*8.6*	**6.6**	**6.0**

[1]Concentrate fed prepartum
[2]Concentrate fed postpartum
[3]Additional control concentrate provided at 1 kg per day per animal to the cows assigned to the group without nicotinic acid treatment from 42 d prepartum to 24 d postpartum
[4]Additional concentrate premix provided at 1 kg per day per animal to the cows assigned to the group receiving nicotinic acid treatment from 42 d prepartum to 24 d postpartum
[5]For dry cows. Ingredients per kg mineral feed: 60 g Ca; 100. 5 g Na; 80 g P; 50 g Mg; 7 g Zn; 4.8 g Mn; 1.3 g Cu; 100 mg I; 40 mg Se; 30 mg Co; 800,000 IU vitamin A; 100,000 IU vitamin D_3; 1500 mg vitamin E
[6]For lactating dairy cows. Ingredients per kg mineral feed: 140 g Ca; 120 g Na; 70 g P; 40 g Mg; 6 g Zn; 5.4 g Mn; 1 g Cu; 100 mg I; 40 mg Se; 25 mg Co; 1,000,000 IU vitamin A; 100,000 IU vitamin D_3; 1,500 mg vitamin E
[7]DM: dry matter

4.1.3. Sample Collection

Prior to biopsy sampling, a blood sample was taken by jugular venipuncture to determine plasma NEFA and glycerol concentrations. Blood samples were centrifuged at 2000×*g* for 15 min at 15°C, and were stored at -80°C until clinical chemical analysis. Measurements were performed with commercial colorimetric kits (NEFA-HR (2), Wako Chemicals GmbH, Neuss, Germany; Free glycerol reagent, Sigma-Aldrich, St. Louis, MO, USA).

Adipose tissue biopsy samples were collected from the SCAT and the RPAT depot 42 d prepartum, and 1, 21, and 100 d postpartum, according to McNamara and Hillers (1986a) and Locher et al. (2011). After preparation of the surgical field and local anesthesia induced with procaine (Procaine 2 %; Selectavet Dr. Otto Fischer GmbH, Weyarn-Holzolling, Germany), samples from adipose tissues were collected under antiseptic conditions (approx. 5 gram tissue per biopsy sample). A 3 cm skin incision was made in the region of the tail head on alternating sides (right and left) to obtain SCAT. For the collection of RPAT, a 3 to 5 cm skin incision was made in the angle between the lumbar transversal processes and the iliac bone, muscles were dissected reaching the peritoneum, and tissue samples were taken directly from the adipose depot localized above the peritoneum. Biopsies of RPAT were obtained each time alternating from the left and right flank. Skin incisions were closed with horizontal interrupted mattress suture pattern (Filovet; Wirtschaftsgenossenschaft Deutscher Tierärzte, Garbsen, Germany). After removal, tissue samples were trimmed of connective and vascular tissue and rinsed thoroughly in physiological saline solution to reduce blood contamination. Samples were divided into 4 parts as follows, according to the 4 different experiments carried out within this PhD research.

One part of each tissue sample was immediately embedded in Tissue Tek (Sakura Finetek Europe, Alphen aan den Rijn, The Netherlands) and snap frozen for later histological preparation (see section *"4.2. Characterization of the Morphological Adaptation of Adipose Tissues"*). A second part of each tissue sample was immediately snap-frozen in liquid nitrogen and stored thereafter at -80°C until Western blot analyses, to detect protein expression levels being present in vivo (see section *"4.3. Analysis of the Extent of Lipolysis and Lipogenesis by the Detection of Marker Proteins"*). A third part of each tissue sample was used to perform an in vitro lipolysis assay detecting lipolytic response to β-adrenergic stimulation (see section *"4.4. Evaluation of In Vitro β-Adrenergic Response of Adipose Tissues"*). Additionally, one further part of the tissues deriving from 10 cows was used to perform an in vitro lipolysis assay to evaluate the antilipolytic effect mediated by the GPR109A-pathway (see section *"4.5. Assessment of the Antilipolytic Effect Mediated by the GPR109A-Pathway"*).

4.2. Characterization of the Morphological Adaptation of Adipose Tissues

4.2.1. Histomorphometric Measurement

Histological sections of SCAT and RPAT samples obtained from 13 out of the 20 cows were prepared using a Leica Jung CM3000 cryostat at -30°C. Tissue samples embedded in Tissue Tek were cut into 10 µm thick sections. Six non-adjacent sections of each sample were mounted onto Superfrost glass slides (Gerhard Menzel GmbH, Braunschweig, Germany). Sections were fixated in 4 % formaldehyde for 10 min and then hematoxylin-eosin stained. Meyer's Hemalaun solution (AppliChem GmbH, Darmstadt, Germany) was applied for 5 min, and 0.25 % eosin solution (Sigma-Aldrich) was applied for 2 min to the sections. After dehydration in ethanol and clearing in xylol, sections were covered with a cover slip attached with Depex (Serva Electrophoresis GmbH, Heidelberg, Germany). Tissue sections were visualized using an Olympus IX70 microscope and digitally captured using a Leica DFC 290 camera. For each sample, cell area (in μm^2) of 164 ± 13 (mean ± SEM) cells was measured in ImageJ 1.48. The median of the measured cell area values within each sample was registered and assigned to the corresponding sample as a single 'cell size' value.

4.2.2. Tissue Composition Analysis

To gain additional information on the structural changes of adipose tissues in the course of the periparturient period, DNA, TG, and total protein content, and β-actin protein expression was quantified in the SCAT and RPAT samples of all 20 cows.

DNA Content. To isolate DNA from the samples, 100 mg of tissue was homogenized in 1 ml of DNAzol reagent (Invitrogen, Life Technologies GmbH Darmstadt, Germany) using an Eppendorf pestle. Homogenates were incubated at 60°C for 5 min with continuous shaking at 350 rpm and briefly ultrasonicated to ensure complete cell lysis. Centrifugation at 10,000 g for 10 min at 4°C was performed in order to remove insoluble tissue fragments. The supernatant was removed to a new tube. DNA was precipitated, washed and then resuspended in 8 mM NaOH solution according to the manufacturer's protocol. Finally, measured DNA concentrations were corrected for wet weight of the tissue sample and results were expressed as µg DNA per g tissue.

Triglyceride Content. Triglyceride content of tissue samples was measured using a colorimetric kit (BioVision Inc, Milpitas, CA, USA). To prepare homogenates, approximately 500 mg tissue was ground in liquid nitrogen. From this tissue powder, a representative aliquot

of 50 mg was weighed and mixed with 1 ml 5 % Nonidet P40 detergent solution (Fluka Feinchemikalien GmbH, Neu-Ulm, Germany). This mixture was further homogenized with a FastPrep-24 tissue homogenizer (MPI Biomedicals, Santa Ana, CA, USA) and incubated at 99°C for 5 min with continuous shaking at 300 rpm. Afterwards, samples were centrifuged at 10,000 g for 2 min. The upper lipid layer and supernatant were transferred into a new tube. Incubation, centrifugation and transfer of the supernatant were repeated to maximize lipid extraction yield. Triglyceride concentration in the lipid extract was measured in duplicate according to the manufacturer's protocol with a spectrophotometer. Finally, measured TG concentrations were corrected for tissue wet weight and results were expressed as mmol TG per g tissue.

Total Protein Content. Total protein concentration was determined based on the Bradford method. To extract proteins, the same tissue powder was used as for the TG content measurement. One hundred gram of this powder was homogenized in a lysis buffer [50 mM HEPES (Carl Roth GmbH, Karlsruhe, Germany), 4 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (Sigma-Aldrich), 10 mM EDTA (Sigma-Aldrich), 0.1 % Triton X-100 (Sigma-Aldrich), 100 mM β-glycerol phosphate (Sigma-Aldrich), 15 mM sodium pyrophosphate (Sigma-Aldrich), 5 mM sodium orthovanadate (Sigma-Aldrich), 2.5 mM sodium fluoride (Sigma-Aldrich) and a protease inhibitor cocktail (CompleteMini, Roche Diagnostics GmbH, Mannheim, Germany)] with a FastPrep-24 tissue homogenizer (MPI Biomedicals). Protein extracts were centrifuged at 10,000 g for 5 min at 4°C. The protein-containing fraction, situated under the lipid layer, was then transferred into a new tube and protein concentration was measured by using Bradford reagent (Serva Electrophoresis GmbH) in triplicate according to the manufacturer's protocol. Finally, measured protein concentrations were corrected for wet weight of the sample and results were expressed as mg total protein per g tissue.

β-Actin Protein Expression. Protein extracts prepared for protein content measurement were used to quantify β-actin expression by Western blotting. Samples were diluted to 0.5 mg total protein per ml in loading buffer [50 mM Tris-HCl (Sigma-Aldrich), 10 % glycerol (Sigma-Aldrich), 2 % SDS (Serva Electrophoresis GmbH), 0.1 % bromophenol blue (Sigma-Aldrich), 2 % mercaptoethanol (Sigma-Aldrich); final concentrations] and heated at 95°C for 5 min. Twenty microliter of the diluted samples were separated by SDS-PAGE on 8.1 % hand-casted gels and transferred to nitrocellulose membranes in duplicate. Membranes were blocked in a PBS-based solution containing 5 % fat-free milk powder (Carl Roth GmbH) and 0.1 % Tween 20 (Sigma-Aldrich) for 1 h at room temperature. Blocked membranes were

incubated for 1 h at room temperature with a mouse monoclonal anti-β-actin antibody (dilution 1:10,000, catalog number A5441, Sigma-Aldrich). Afterwards, membranes were incubated with a peroxidase conjugate anti-mouse secondary antibody (catalog number A2304, dilution 1:50,000, Sigma-Aldrich) at room temperature for 1 hour. Immunodetection was performed by incubating the membranes with LumiGlo reagent (Cell Signaling Technology, Danvers, MA) and chemiluminescence was detected by a ChemiDoc XRS+ system (Bio-Rad Laboratories GmbH, München, Germany). Bands were quantified by densitometry using Image Lab 4.0 software (Bio-Rad Laboratories GmbH). Finally, membranes were Indian ink stained (Pelikan PBS, Peine, Germany) to control equal loading.

4.3. Analysis of the Extent of Lipolysis and Lipogenesis by the Detection of Marker Proteins

4.3.1. Sample Preparation

Biopsy samples of SCAT and RPAT received from all 20 cows at 42 d prepartum, and 1, 21, and 100 d postpartum were used to quantify the extent of expression and phosphorylation of key proteins involved in adipose lipolytic and lipogenic pathways. Samples were prepared for Western blot analyses as follows. Protein extraction was performed in a lysis buffer containing 50 mM HEPES (Carl Roth GmbH), 4 mM ethylene glycol-bis(2-aminoethylether)-N,N,N’,N’-tetraacetic acid (Sigma-Aldrich), 10 mM EDTA (Sigma-Aldrich), 0.1 % Triton X-100 (Sigma-Aldrich), 100 mM β-glycerol phosphate (Sigma-Aldrich), 15 mM sodium pyrophosphate (Sigma-Aldrich), 5 mM sodium orthovanadate (Sigma-Aldrich), 2.5 mM sodium fluoride (Sigma-Aldrich), a protease inhibitor cocktail (CompleteMini, Roche Diagnostics GmbH), and a phosphatase inhibitor cocktail (PhosStop, Roche Diagnostics GmbH) with a FastPrep-24 tissue homogenizer (MPI Biomedicals). Extracts were centrifuged at 10,000×g for 10 min at 4°C, and aliquots of the supernatants were stored at -20°C until electrophoresis. Protein concentrations were measured using Bradford reagent (Serva Electrophoresis GmbH). Protein extracts were diluted to 0.5 mg/ml in loading buffer (50 mM Tris-HCl [Sigma-Aldrich], 10 % glycerol [Sigma-Aldrich], 2 % SDS [Serva Electrophoresis GmbH], 0.1 % bromophenol blue [Sigma-Aldrich], 2 % mercaptoethanol [Sigma-Aldrich]; final concentrations) and heated at 95°C for 5 min. Ten micrograms protein per lane were separated by SDS-PAGE on 8.1 % hand-casted gels and transferred to nitrocellulose membranes. Membranes were blocked in a PBS-based solution

containing 5 % fat-free milk powder (Carl Roth GmbH) and 0.1 % Tween 20 (Sigma-Aldrich) for 1 hour at room temperature.

4.3.2. Measurement of Protein Expression and Phosphorylation: HSL, Perilipin and FAS

Two measures of adipocyte lipolysis were HSL phosphorylation and perilipin phosphorylation. Also the total protein abundance of HSL and perilipin was quantified. Lipogenesis was assessed by the protein abundance of FAS. Technically, blocked membranes were incubated overnight at 4°C with the corresponding primary antibodies: total HSL (dilution 1:5,000, catalog number 4107, Cell Signaling Technology), HSL phosphorylated at Ser-563 (dilution 1:1,000, catalog number 4139, Cell Signaling Technology), total perilipin (dilution 1:2,000, catalog number AB10200, Chemicon International, Temecula, CA), perilipin phosphorylated at Ser-522 (dilution 1:5,000, catalog number 4856, Vala Sciences, San Diego, CA), FAS (dilution 1:500, catalog number F9554, Sigma-Aldrich). Afterwards, membranes were incubated with the matching secondary antibody (horseradish peroxidase **(HRP)**-linked anti-rabbit IgG, dilution 1:2,500, catalog number 7074, Cell Signaling Technology or peroxidase conjugate anti-mouse IgG, dilution 1:50,000, catalog number A2304, Sigma-Aldrich) at room temperature for 1 hour. Immunodetection was performed by incubating the membranes with LumiGlo reagent (Cell Signaling Technology) or WesternBright Sirius (Advansta Corporation, Menlo Park, CA) and chemiluminescence was detected by a ChemiDoc XRS+ system (Bio-Rad Laboratories GmbH). Bands were quantified by densitometry using Image Lab 4.0 software (Bio-Rad Laboratories GmbH). Finally, membranes were Indian ink stained (Pelikan PBS) to control equal loading. Quantities of phospho-HSL, and phospho-perilipin were normalized for total quantity of the corresponding protein detected in the same sample. These normalized values are referred to as “extent of phosphorylation” throughout the text.

4.4. Evaluation of In Vitro β-Adrenergic Response of Adipose Tissues

4.4.1. Lipolysis Assay and Measurement of Functional Response

Biopsy samples of SCAT and RPAT deriving from all 20 cows at 42 d prepartum, and 1, 21, and 100 d postpartum were processed in an in vitro lipolysis assay to assess lipolytic response at different stages of the periparturient period. Lipolytic response was triggered with the β-adrenergic agonist isoproterenol, according to the method of McNamara and Hillers

(1986a) with modifications described below. Immediately after sampling, tissue samples (approx. 100 mg) were pre-incubated in Dulbecco's Modified Eagle's Medium **(DMEM)** (Life Technologies Corporation, Paisley, UK) containing 2 % fatty acid free bovine serum albumin **(BSA)** (Sigma-Aldrich) for 20 min at 37°C to diminish unspecific release of metabolites due to handling and cutting. Each sample was then incubated separately in 1 ml of freshly prepared DMEM containing 2 % fatty acid free BSA (incubation medium) for 90 min at 37°C with gentle shaking. Incubation medium was supplemented with 0 or 1 μM isoproterenol (Sigma-Aldrich) to establish incubation sets with basal lipolytic conditions or with induced lipolytic conditions, respectively. Incubation sets were performed in triplicate. Following incubation, tissue samples were briefly drained, snap-frozen in liquid nitrogen, weighed and afterwards stored at -80°C until Western blot analyses. Incubation media were stored at -20°C until further biochemical analysis.

To assess lipolytic response of the incubated tissue samples, glycerol concentration (Free glycerol reagent, Sigma-Aldrich) and NEFA concentration (NEFA-HR (2), Wako Chemicals GmbH) in the incubation media were measured. Concentrations of glycerol and NEFA were corrected for wet weight and expressed as μmol metabolite per g tissue per 90 minutes of incubation.

4.4.2. Measurement of Protein Expression and Phosphorylation: HSL, Perilipin and β_2-Adrenergic Receptor

In addition to measuring NEFA and glycerol release, the extent of HSL phosphorylation and perilipin phosphorylation were also detected in the incubated tissue samples to monitor the effect of β-adrenergic stimulation on the activation of lipolytic proteins. Tissue samples of the triplicate incubations were pooled together for protein detection. Preparation of the samples for Western blot and detection of phosphorylated HSL and perilipin was performed the same way as in the non-treated tissues (described above in *"4.3. Analysis of the Extent of Lipolysis and Lipogenesis by the Detection of Marker Proteins"*). Furthermore, to detect changes in β_2-adrenergic receptor expression and desensitization throughout the periparturient period, the total expression of the receptor protein and its phosphorylation was measured in non-incubated samples. Technically, blocked membranes were incubated overnight at 4°C with the corresponding primary antibodies: total β_2-adrenergic receptor (dilution 1:600, catalog number 569, Santa Cruz Biotechnology, Santa Cruz, CA), and β_2-adrenergic receptor phosphorylated at Ser-345/Ser-346 (dilution 1:600, catalog number 16718-R, Santa Cruz

Biotechnology). Afterwards, membranes were incubated with the matching secondary antibody (anti-rabbit IgG, HRP-linked, dilution 1:2,500, Cell Signaling Technology) at room temperature for 1 hour. Immunodetection was performed by incubating the membranes with WesternBright Sirius (Advansta Corporation) and chemiluminescence was detected by a ChemiDoc XRS+ system (Bio-Rad Laboratories GmbH). Bands were quantified by densitometry using Image Lab 4.0 software (Bio-Rad Laboratories GmbH). Finally, membranes were Indian ink stained (Pelikan PBS) to control equal loading. Quantities of phospho-β_2-adrenergic receptor were normalized for total quantity of the receptor protein detected in the same sample. These normalized values are referred to as "extent of phosphorylation" throughout the text.

4.5. Assessment of the Antilipolytic Effect Mediated by the GPR109A-Pathway

4.5.1. Lipolysis Assay and Measurement of Functional Response

To evaluate the antilipolytic effect of the GPR109A-mediated pathway, an in vitro lipolysis assay was performed, similarly as described for the evaluation of the β-adrenergic response of adipose tissues (see chapter 4.4.1). However, in this case, ligands of GPR109A were added to the induced lipolytic incubation sets as follows. Triplicates of SCAT and RPAT tissue samples were incubated immediately after sampling in the presence of NAC (n=6) or NAM (n=6). These tissue samples were also deriving from the animals described in chapter 4.1.1. Cows belonging to the group without dietary NAC supplementation, being at various stages of lactation were selected for this in vitro assay. Altogether samples of 10 cows were used, such that samples of 2 cows were used both for incubation with NAC and with NAM.

First, tissues were pre-incubated in DMEM (Life Technologies Corporation) containing 2 % fatty acid free BSA (Sigma-Aldrich) for 20 min at 37°C to diminish unspecific release of metabolites due to handling and cutting. Each sample (about 100 mg) was then incubated separately in 1 ml of freshly prepared DMEM containing 2 % fatty acid free BSA (incubation medium) for 90 min at 37°C with gentle shaking. Incubation medium was supplemented with 0 or 1 μM isoproterenol (Sigma-Aldrich) to establish incubation sets with basal lipolytic conditions or with induced lipolytic conditions, respectively. In addition to basal and isoproterenol-stimulated incubation sets, antilipolytic incubation sets with NAC or with NAM were applied. These antilipolytic sets included 1 μM isoproterenol and 2 μM, 8 μM or 32 μM

NAC or NAM. The concentrations of NAC and NAM were chosen to match physiologically occurring concentrations in bovine blood plasma (Niehoff et al., 2009). Following incubation, tissue samples were briefly drained, then weighed and afterwards stored at -80°C until Western blot analysis. Incubation media were stored at -20°C until further biochemical analysis.

To assess lipolytic response of the incubated tissue samples, glycerol concentration (Free glycerol reagent, Sigma-Aldrich) and NEFA concentration (NEFA-HR(2), Wako Chemicals GmbH) in the incubation media were measured. Concentrations of glycerol and NEFA were corrected for wet weight (μmol metabolite per g tissue per 90 minutes of incubation). Glycerol and NEFA release of samples without stimulation (basal lipolysis) were compared to the release of samples treated with isoproterenol (induced lipolysis) to assess pro-lipolytic responsiveness of tissue explants. Glycerol and NEFA release of samples treated with the combination of isoproterenol and NAC or NAM were compared to the release of isoproterenol-treated samples and the difference was considered as the antilipolytic effect of the applied agent.

4.5.2. Measurement of Protein Expression and Phosphorylation: HSL and GPR109A

The technical replicates of the triplicate tissue incubation were pooled together, and about 200 mg of these samples were ground in 0.7 ml lysis buffer [50mM HEPES (Carl Roth GmbH), 4 mM ethylene glycol-bis(2-aminoethylether)-N,N,N',N'-tetraacetic acid (Sigma-Aldrich), 10 mM EDTA (Sigma-Aldrich), 0.1 % Triton X-100 (Sigma-Aldrich), 100 mM β-glycerol phosphate (Sigma-Aldrich), 15 mM sodium pyrophosphate (Sigma-Aldrich), 5 mM sodium orthovanadate (Sigma-Aldrich), 2.5 mM sodium fluoride (Sigma-Aldrich), protease inhibitor (CompleteMini, Roche Diagnostics GmbH), phosphatase inhibitor (PhosStop, Roche Diagnostics GmbH)] and incubated for 2 hours at 4°C with gentle shaking. Mixtures were subsequently homogenized using a 22-gauge needle and finally centrifuged at 1000×*g* for 10 min at 4°C. Aliquots of the supernatants were stored at -20°C until electrophoresis. Protein concentration in the supernatants was measured using Bradford reagent (Serva Electrophoresis GmbH).

Protein extracts were diluted to 0.5 mg/ml in loading buffer [50 mM Tris-HCl (Sigma-Aldrich), 10 % glycerol (Sigma-Aldrich), 2 % SDS (Serva Electrophoresis GmbH), 0.1 % bromophenol blue (Sigma-Aldrich), 2 % mercaptoethanol (Sigma-Aldrich); final concentrations] and heated at 95°C for 5 min. Ten micrograms of protein per lane were

separated by SDS-PAGE on 8.1 % hand-casted gels and transferred to nitrocellulose membranes. Membranes were blocked in PBS solution containing 5 % fat-free milk powder (Carl Roth GmbH) and 0.1 % Tween 20 (Sigma-Aldrich) for 1 hour at room temperature and then incubated with an antibody against phosphorylated HSL at Ser-563 (dilution 1:1,000, catalog number 4139, Cell Signaling Technology), overnight at 4°C. Membranes were incubated with a corresponding secondary antibody (anti-rabbit-HRP, dilution 1:2,500, catalog number 7074, Cell Signaling Technology) for 1 hour at room temperature. Immunodetection was performed by incubating the membranes with LumiGlo reagent (Cell Signaling Technology) and chemiluminescence was detected by a ChemiDoc XRS+ system (Bio-Rad Laboratories GmbH). Bands were quantified by densitometry using Image Lab 4.0 software (Bio-Rad Laboratories GmbH). Finally, membranes were Indian ink stained (Pelikan PBS) to control equal loading.

Membranes containing samples of basal incubation sets were additionally incubated with an anti-HM74 (homologous to GPR109A) antibody (dilution 1:100, catalog number 48537, Santa Cruz Biotechnology) overnight at 4°C and afterwards with an anti-goat-HRP secondary antibody (dilution 1:80,000, catalog number A5420, Sigma-Aldrich) for 1 hour at room temperature. The anti-HM74 antibody was already used by Titgemeyer et al. (2011) to detect GPR109A receptor protein abundance in bovine adipose tissues. Membranes were incubated with SuperSignal West Dura substrate (Thermo Scientific, Rockford, IL), and chemiluminescence was detected by a ChemiDoc XRS+ system (Bio-Rad Laboratories GmbH). Bands were quantified by densitometry using Image Lab 4.0 software (Bio-Rad Laboratories GmbH). Finally, membranes were Indian ink stained (Pelikan PBS) to control equal loading.

4.6. Statistical Analyses

Evaluated effects were considered to be significant when P values were ≤ 0.05. All statistical tests were performed in GraphPad Prism 6.0. Indicative animal production performance data (dry matter intake **(DMI)**, milk yield, net energy balance, plasma NEFA and glycerol concentration) were analyzed both for time-related effects and for dietary effects by two-way ANOVA.

In each dataset of the experiments described in chapter 4.2, 4.3 and 4.4, the effects of dietary interventions (LC without NAC, LC with NAC, HC without NAC, HC with NAC) were tested by ANOVA. Since no significant differences were detected between these dietary

groups, data were pooled together and further analyzed for the whole group of animals (n=20). The main effects of time related to parturition (42 d prepartum, and 1, 21, and 100 d postpartum) and adipose depot (SCAT, RPAT) were evaluated by two-way ANOVA, also considering interactions. Data were tested for normal distribution by the Shapiro-Wilk test before performing ANOVA. Multiple comparisons between time points were done by Tukey's post-test. In detail, ANOVA was applied to data of cell size, tissue DNA, TG and protein content, β-actin expression, HSL and perilipin expression and phosphorylation both in non-incubated and in in vitro incubated tissues, FAS expression, glycerol and NEFA release of in vitro incubated tissues (using the mean of the triplicate measurements), and β_2-adrenergic receptor expression and phosphorylation.

Cell size data (experiment described in 4.2.1) were analyzed for relative frequency distribution, so that cells were classified as small (under 3,500 μm^2), medium (3,500-7,500 μm^2), big (7,500-11,500 μm^2), or large (over 11,500 μm^2). The distribution pattern between time points were analyzed both in SCAT and in RPAT by one-way ANOVA with a Tukey's multiple comparison test.

To confirm the functional link between protein phosphorylation and lipolytic release, the correlation (Pearson's correlation) between in vitro HSL phosphorylation, perilipin phosphorylation, NEFA release, and glycerol release was calculated (experiment described in 4.4). This was done separately for basal incubation conditions and isoproterenol-stimulated incubation conditions, both for SCAT and for RPAT, collectively for all time points.

To analyze the data of the in vitro experiment investigating the antilipolytic effects of NAC and NAM (described in 4.5), the mean of the triplicate measurements were used. Glycerol release, NEFA release and HSL phosphorylation of basal and isoproterenol-stimulated incubation sets were compared using Student's t-test in both treatment groups (NAC, NAM) in both tissues (SCAT, RPAT). Within treatment groups, glycerol release, NEFA release and HSL phosphorylation of incubation sets with isoproterenol treatment and with antilipolytic treatment were analyzed by two-way ANOVA for factor 'tissue' (SCAT, RPAT) and factor 'treatment' (effect of increasing concentrations of NAC or NAM) with interactions. Tukey's post-test was performed for multiple comparisons. GPR109A protein expression was compared between SCAT and RPAT using Student's t-test.

5. Results

5.1. Animal Production Performance as Influenced by Dietary Interventions

Indicative data on DMI, milk yield, net energy balance and plasma NEFA and glycerol concentration of the 20 cows included in the current study, grouped according to feeding regimens at corresponding time points to biopsy samplings are shown in **Fig. 4** and **Fig. 5**.

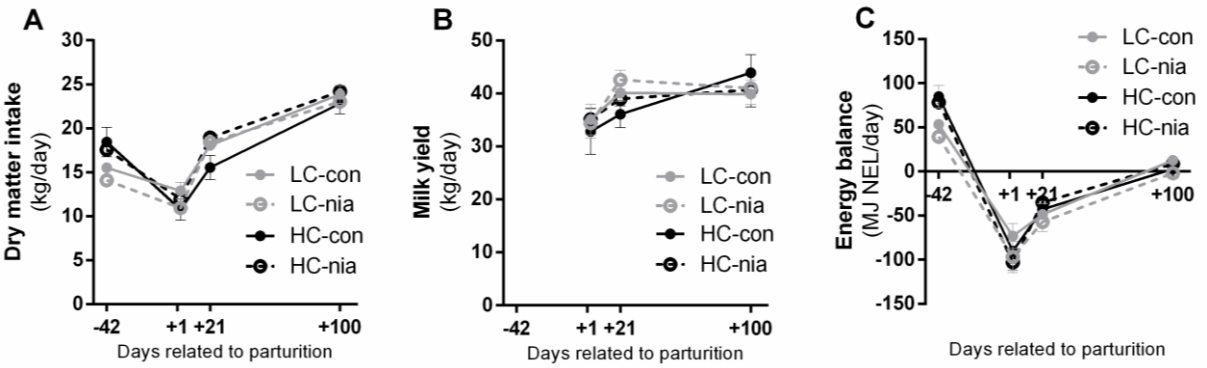

Figure 4. (A) Dry matter intake, **(B)** milk yield, and **(C)** net energy balance shown separately for the feeding groups at corresponding time points to biopsy samplings. (LC-con = low concentrate proportion, no nicotinic acid supplementation; LC-nia = low concentrate proportion, with nicotinic acid supplementation; HC-con = high concentrate proportion, no nicotinic acid supplementation; HC-nia = high concentrate proportion, with nicotinic acid supplementation). Means ± SEM, n = 20.

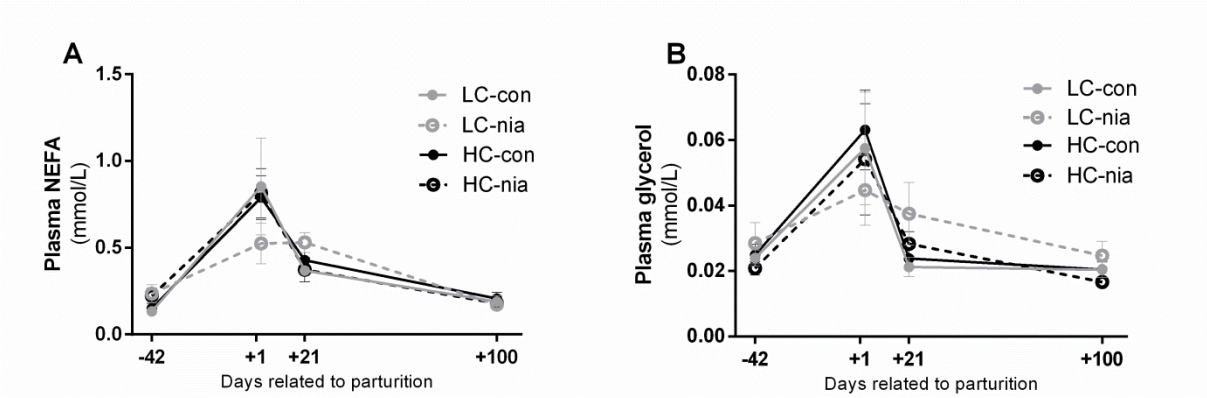

Figure 5. (A) Plasma NEFA, and **(B)** plasma glycerol concentration shown separately for the feeding groups at corresponding time points to biopsy samplings. (LC-con = low concentrate proportion, no nicotinic acid supplementation; LC-nia = low concentrate proportion, with nicotinic acid supplementation; HC-con = high concentrate proportion, no nicotinic acid supplementation; HC-nia = high concentrate proportion, with nicotinic acid supplementation). Means ± SEM, n = 20.

Time-related changes were highly significant for each of the data presented in **Fig. 4** and **Fig. 5** ($P < 0.001$); however, feeding groups of different concentrate proportions and NAC supplementation did not significantly differ. Moreover, all other variables investigated in this PhD research remained unaffected by dietary manipulations. Therefore, dietary groups were pooled together, and further results are presented collectively for all 20 cows.

5.2. Morphological Adaptation of Adipose Tissues during the Periparturient Period

5.2.1. Changes in Adipose Cell Size

Adipocytes had a smaller cell size at the end of the trial compared to the beginning ($P < 0.001$) as shown in **Fig. 6**. However, the pattern of the differences was not exactly the same in SCAT and RPAT, resulting in a significant interaction ($P < 0.01$) between time course and tissue depot. In fact, SCAT cells were found to be gradually smaller at the 4 studied time points, whereas RPAT cells were bigger at 1 d postpartum compared with 42 d prepartum, and were thereafter smaller at 21 d and 100 d postpartum. These time- and tissue-related differences were also reflected in the frequency distribution pattern of the cell size at different time points (**Fig. 7A** for SCAT and **Fig. 7B** for RPAT). Small cells occurred more frequently at 100 d postpartum ($P < 0.001$ in RPAT), while the highest number of large cells was observed at 42 d prepartum in SCAT and at 1 d postpartum in RPAT ($P < 0.001$ in RPAT).

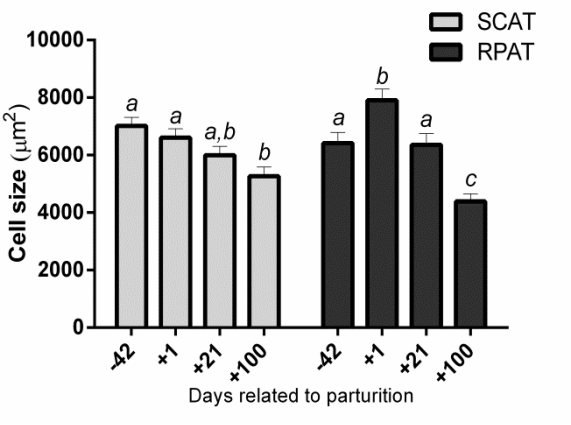

Figure 6. Cell size in subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT) during the periparturient period. Different superscripts indicate $P < 0.05$. Means ± SEM, n = 13.

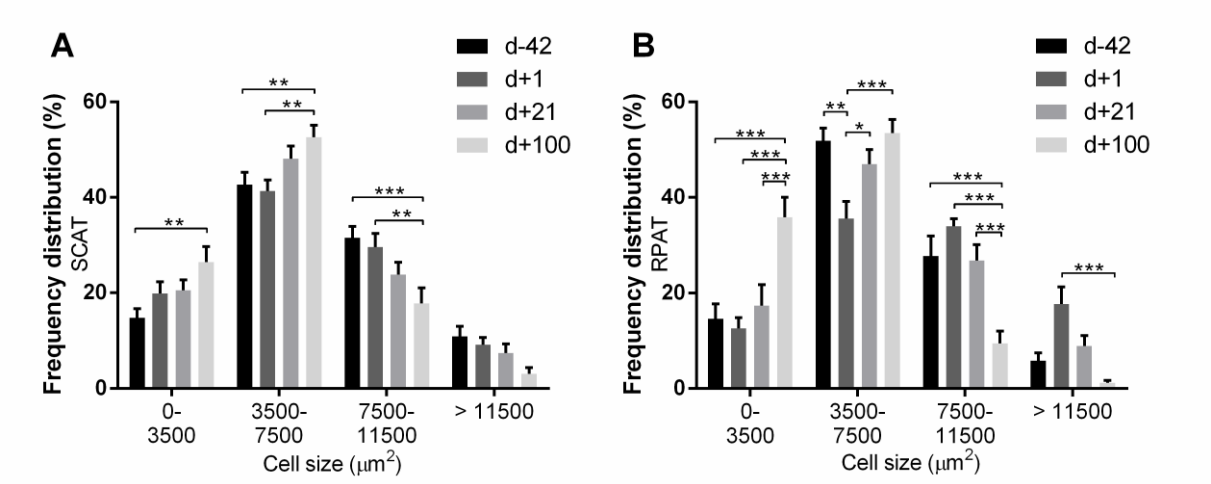

Figure 7. Relative frequency distribution of measured cell size values at different time points during the periparturient period in **(A)** subcutaneous (SCAT) and **(B)** retroperitoneal adipose tissue (RPAT). *P < 0.05, **P < 0.01, ***P < 0.001. Means ± SEM, n = 13.

Exemplary pictures of the hematoxylin and eosin stained histological slides of a representative animal are shown in **Fig. 8**.

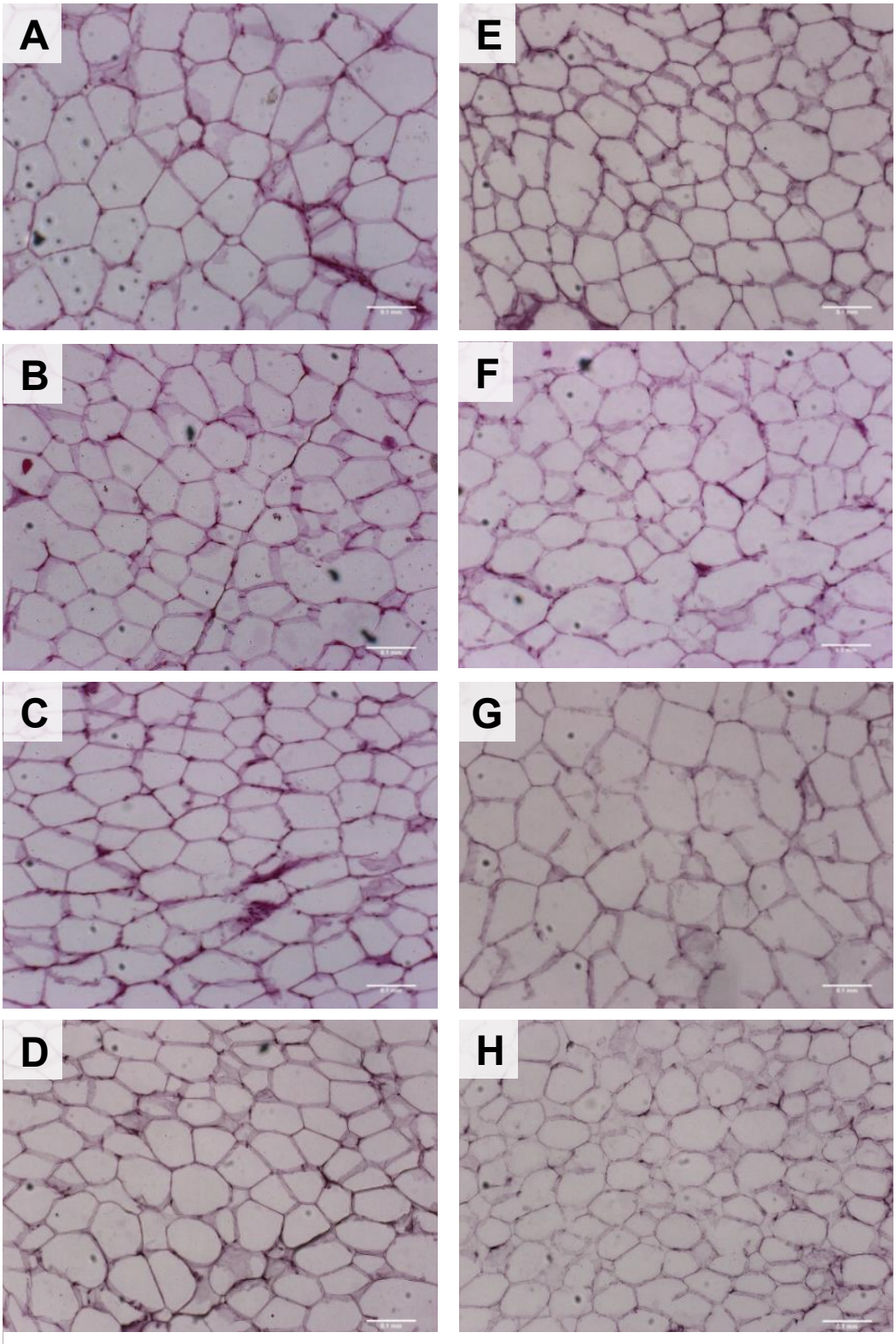

Figure 8. Hematoxylin and eosin stained histological sections of **(A-D)** subcutaneous adipose tissue and **(E-H)** retroperitoneal adipose tissue samples of one representative cows at **(A,E)** 42 days prepartum, and at **(B,F)** 1 day, **(C,G)** 21 days, and **(D,H)** 100 days postpartum. The white measure bar in the lower right corner indicates 100 µm.

5.2.2. Changes in Adipose Tissue Composition

DNA quantification revealed that DNA content of RPAT was greater 100 d postpartum than during early lactation ($P < 0.05$) as shown in **Fig. 9A**. However, SCAT did not have significantly different DNA contents at the observed time points. Triglyceride content per tissue wet weight was not significantly different between the observed time points (**Fig. 9B**). However, there was a remarkable difference between the depots, as RPAT had a lower TG content than SCAT ($P < 0.001$). Total protein content was lower at the postpartum time points in SCAT ($P < 0.05$), and was lower at d 21 postpartum in RPAT compared to the other time points ($P < 0.01$) as shown in **Fig. 9C**. Considering the whole studied period, total protein content was greater in RPAT than in SCAT ($P < 0.001$). As shown in **Fig. 9D**, expression of the cytoskeletal protein β-actin was becoming gradually higher at the observed time points in SCAT ($P < 0.001$) and was significantly higher in SCAT than in RPAT ($P < 0.001$).

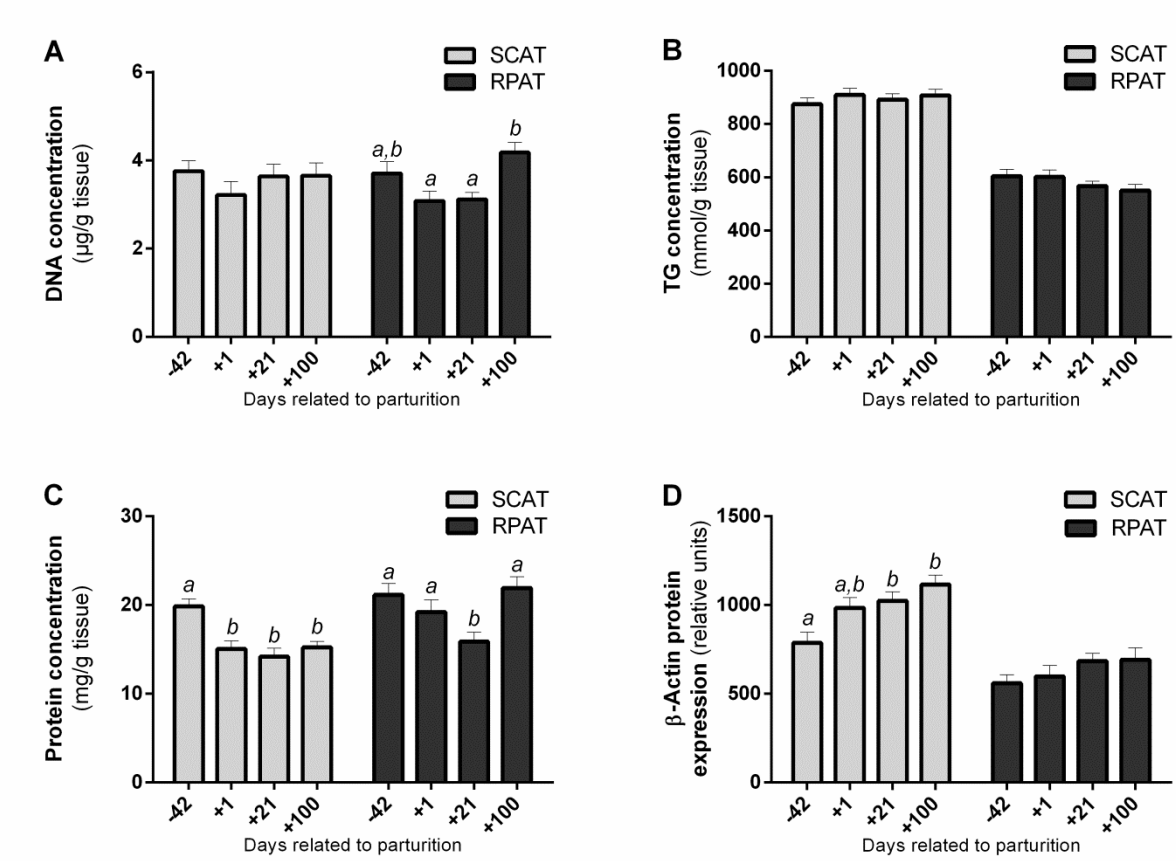

Figure 9. Composition of subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT) samples during the periparturient period. **(A)** DNA content, **(B)** triglyceride (TG) content, **(C)** total protein content, and **(D)** β-actin protein expression. Different superscripts indicate $P < 0.05$. Means ± SEM, n = 20.

5.3. Extent of Lipolysis and Lipogenesis during the Periparturient Period

5.3.1. Lipolysis

Total protein expression of HSL (**Fig. 10A**) and perilipin (**Fig. 10C**) showed only minor variation throughout the experimental period, with the exception of 21 d postpartum in RPAT where HSL expression was higher than at all other time points ($P < 0.05$). In contrast, the extent of HSL and perilipin phosphorylation varied significantly over time ($P < 0.001$), especially in comparison of the prepartum and postpartum period, as shown in **Fig. 10B** and **Fig. 10D**. Phosphorylation was the lowest at 42 d prepartum in case of both proteins in SCAT and in RPAT as well. After parturition, a remarkably higher extent of phosphorylation of these proteins was observed.

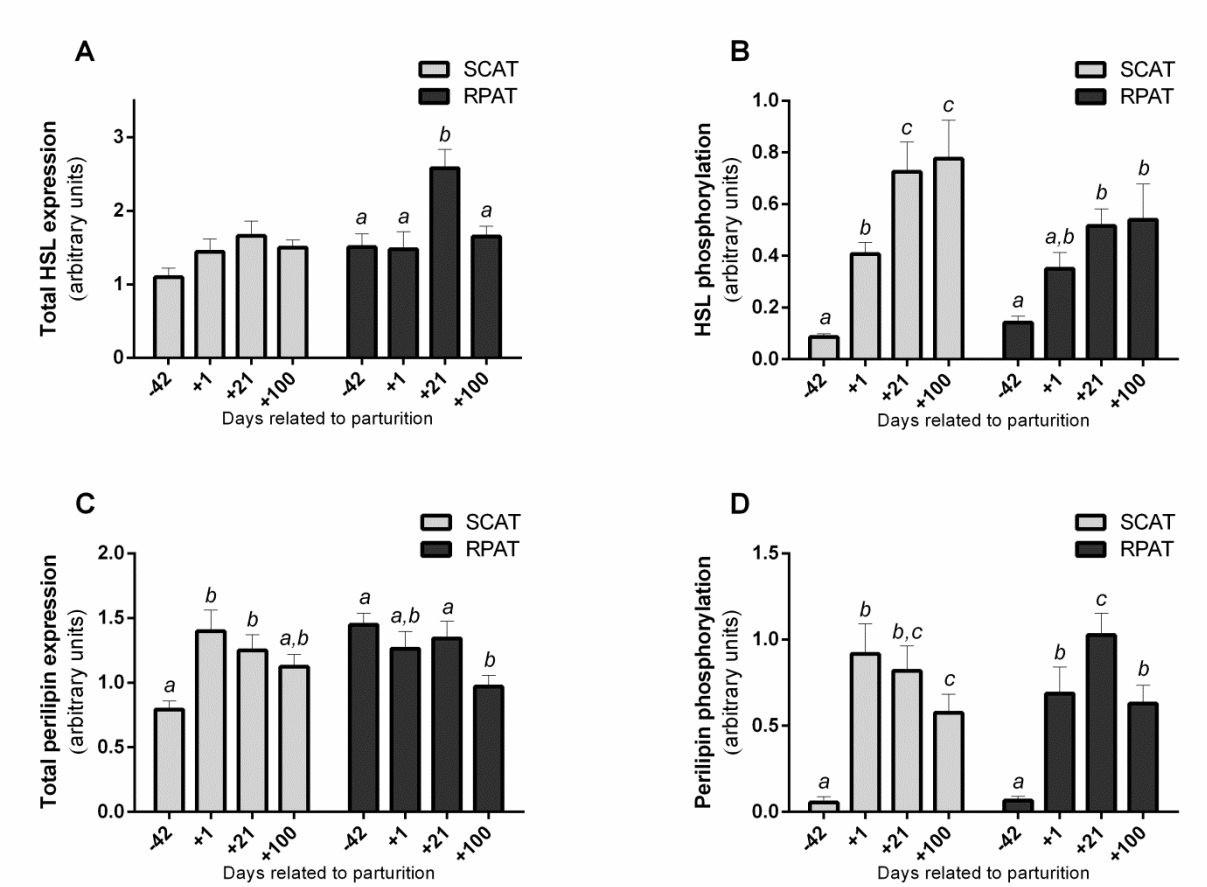

Figure 10. Total expression of **(A)** hormone-sensitive lipase (HSL) and **(C)** perilipin and extent of phosphorylation of **(B)** HSL and **(D)** perilipin in fresh, non-incubated samples of subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT) during the periparturient period. Different superscripts indicate $P < 0.05$. Means ± SEM, n = 20.

5.3.2. Lipogenesis

Fatty acid synthase protein expression was decreased at 1 d postpartum compared to 42 d prepartum, and further decreased at 21 d postpartum both in SCAT and RPAT ($P < 0.001$) as shown in **Fig. 11**. At 100 d postpartum expression nearly returned to the prepartum level.

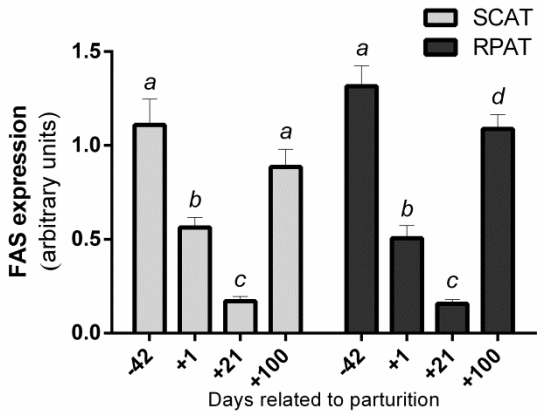

Figure 11. Total expression of fatty acid synthase (FAS) in fresh, non-incubated samples of subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT) during the periparturient period. Different superscripts indicate $P < 0.05$. Means ± SEM, n = 20.

5.4. β-Adrenergic Response of Adipose Tissues during the Periparturient Period

5.4.1. Basal Lipolysis

Basal release of glycerol was significantly affected by the periparturient time ($P < 0.001$), as release rates were greater at 42 d prepartum than after parturition both in SCAT and in RPAT as shown in **Fig. 12A**. NEFA release in the basal incubation sets showed differences over time only in RPAT ($P < 0.001$), as release rates during early lactation were higher than at 42 d prepartum and 100 d postpartum (**Fig. 12B**). Extent of HSL phosphorylation (**Fig. 12C**) was considerably low in the basal incubation set at all time points; nevertheless, it did show some significant variation over time ($P = 0.03$). In case of perilipin, basal incubation did not provide as markedly low phosphorylation as seen in the case of HSL. As shown in **Fig. 12D**, basal phosphorylation was slightly increased during early lactation (differences between time points were statistically not significant).

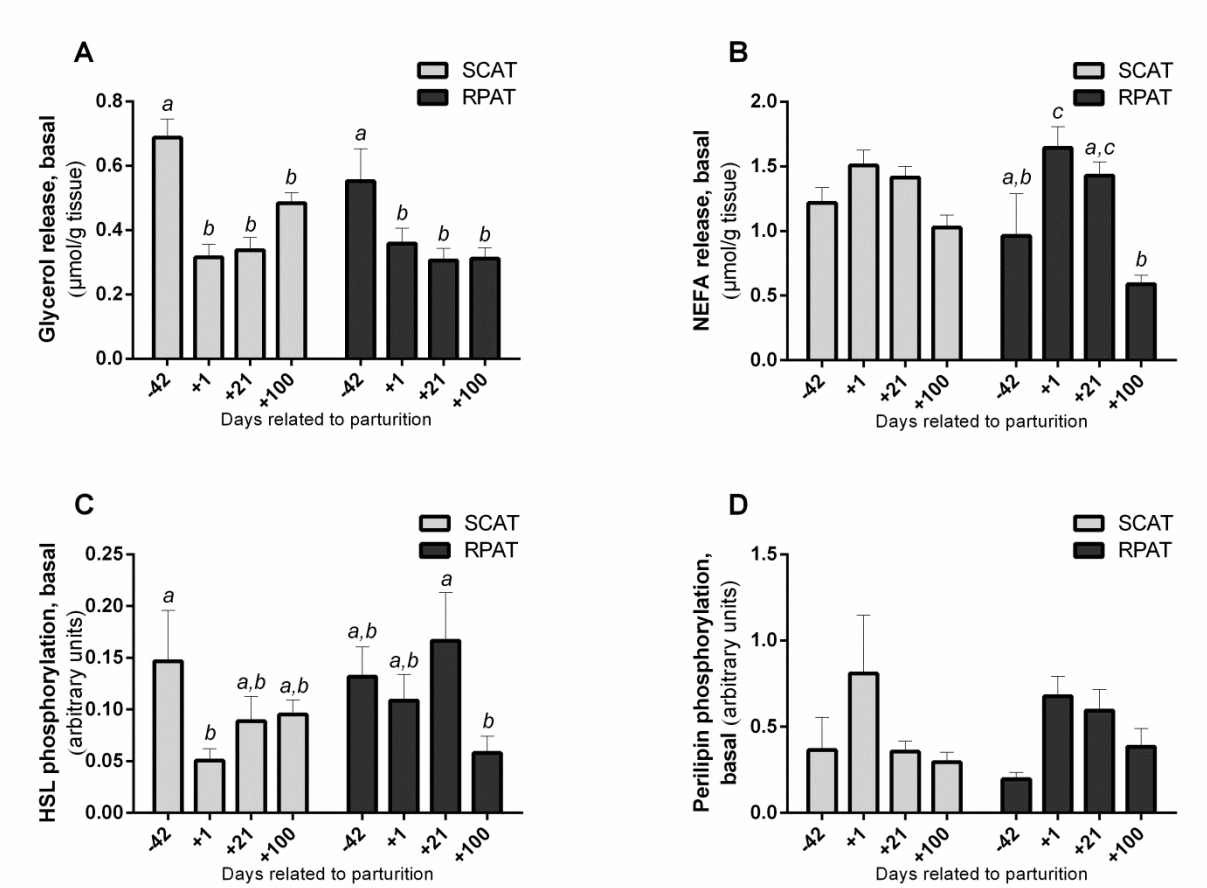

Figure 12. Release of **(A)** glycerol and **(B)** NEFA and extent of **(C)** hormone-sensitive lipase (HSL) phosphorylation and **(B)** perilipin phosphorylation under *basal* lipolytic conditions in in vitro incubated samples of subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT) during the periparturient period. Different superscripts indicate $P < 0.05$. Means ± SEM, n = 20.

5.4.2. Isoproterenol-Stimulated Lipolysis

Isoproterenol-treated samples released the greatest amount of glycerol at 42 d prepartum (**Fig. 13A**). Besides a significant time-effect ($P < 0.001$), a tissue-effect was detected as well ($P = 0.005$), and a significant interaction was also found between time and tissue ($P = 0.01$), reflecting that RPAT released more glycerol than SCAT at 42 d prepartum, but postpartum this difference was not perpetuated. Isoproterenol treatment also triggered the greatest NEFA release at 42 d prepartum (**Fig. 13B**). Besides the time-dependent differences ($P < 0.001$), a tissue-effect was also detected ($P < 0.001$) as RPAT had greater release rates than SCAT prepartum. Also a significant interaction between time and tissue was found ($P = 0.001$) similarly to the dynamics seen in glycerol release. Isoproterenol treatment triggered the greatest extent of HSL phosphorylation at 42 d prepartum, whereas values of 1, 21, and 100 d postpartum were remarkably lower (**Fig. 13C**). Time-specific ($P < 0.001$) but no tissue-

specific differences could be observed in HSL phosphorylation of the isoproterenol-treated tissues. In case of perilipin, isoproterenol-stimulated phosphorylation was the greatest at 42 d prepartum in SCAT (**Fig. 13C**). Lower phosphorylation extent was observed postpartum in SCAT. In RPAT no significant differences in perilipin phosphorylation were observed.

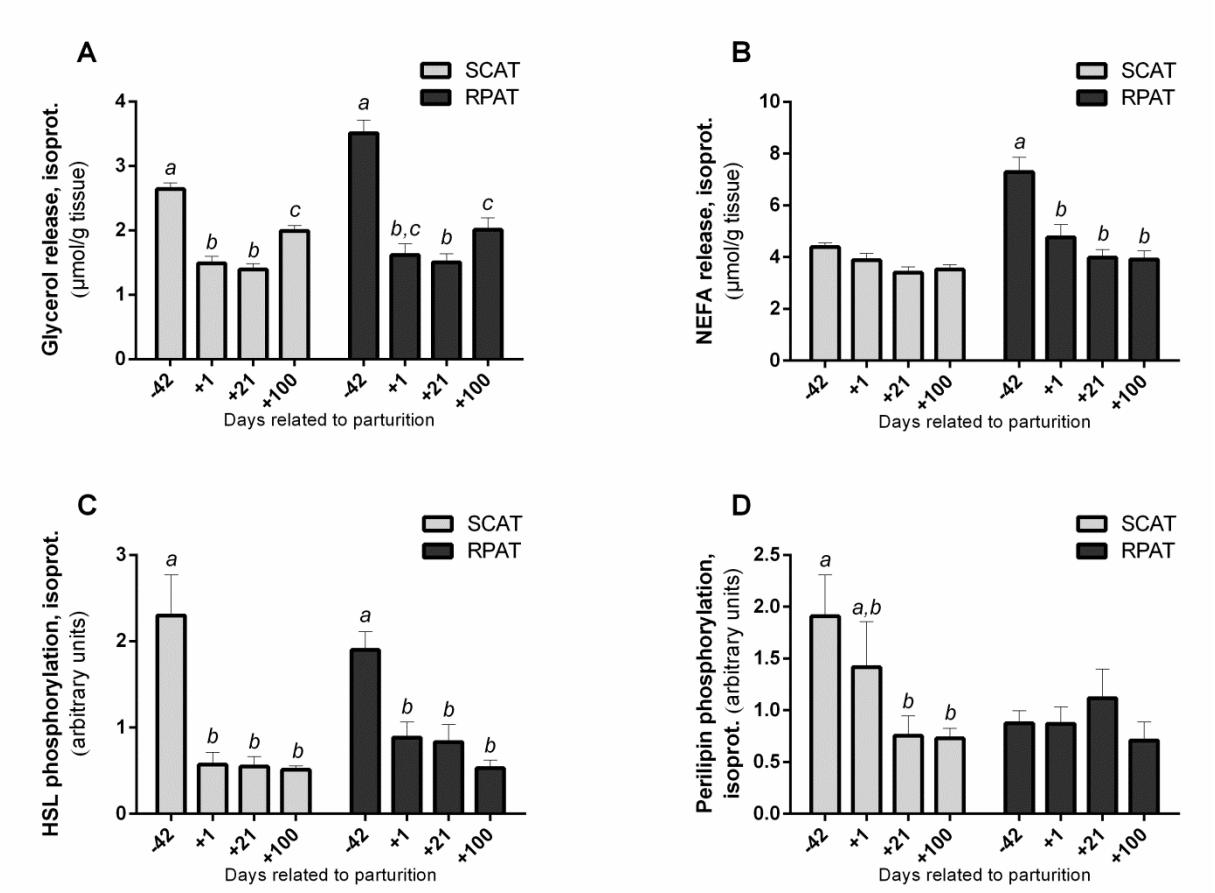

Figure 13. Release of **(A)** glycerol and **(B)** NEFA and extent of **(C)** hormone-sensitive lipase (HSL) phosphorylation and **(B)** perilipin phosphorylation under *isoproterenol-stimulated* lipolytic conditions in in vitro incubated samples of subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT) during the periparturient period. Different superscripts indicate $P < 0.05$. Means ± SEM, n = 20.

Under basal incubation conditions, many of the measures of in vitro lipolysis were correlated with each other. Under isoproterenol treatment all studied measures were correlated with a high significance (**Table 2**).

Table 2. Correlation coefficients (r) for the extent of hormone-sensitive lipase and perilipin phosphorylation (HSL-P and Perilipin-P, respectively) and NEFA and glycerol release under basal and isoproterenol-stimulated incubation conditions (collectively for 42 d prepartum, and 1, 21, and 100 d postpartum). Coefficients above the diagonal stand for subcutaneous adipose tissue, and below for retroperitoneal adipose tissue.

Tissues incubated under basal conditions				
	HSL-P	**Perilipin-P**	**NEFA**	**Glycerol**
HSL-P	-	0.01	0.17	0.46^{***}
Perilipin-P	0.23^{*}	-	0.15	0.03
NEFA	0.32^{**}	0.31^{**}	-	0.33^{**}
Glycerol	0.57^{***}	-0.04	$0.21^{\dagger}$	-

Tissues with induced lipolysis (incubation with 1 μM isoproterenol)				
	HSL-P	**Perilipin-P**	**NEFA**	**Glycerol**
HSL-P	-	0.33^{**}	0.37^{***}	0.54^{***}
Perilipin-P	0.43^{***}	-	0.39^{***}	0.55^{***}
NEFA	0.53^{***}	0.47^{***}	-	0.66^{***}
Glycerol	0.54^{***}	0.42^{***}	0.86^{***}	-

$^{\dagger}P < 0.1$; $^{*}P < 0.05$; $^{**}P < 0.01$; $^{***}P < 0.001$

5.4.3. β-Adrenergic Receptor Protein Expression and Phosphorylation

The total protein expression of β_2-aderenergic receptor was significantly lower at 1 d postpartum than at any other investigated time, both in SCAT and in RPAT ($P < 0.001$) as shown in **Fig. 14A**. At the same time, the extent of receptor phosphorylation was the greatest ($P < 0.001$) as shown in **Fig. 14B**. Protein expression was greater again later postpartum, and protein phosphorylation also returned to nearly the same level which was observed prepartum.

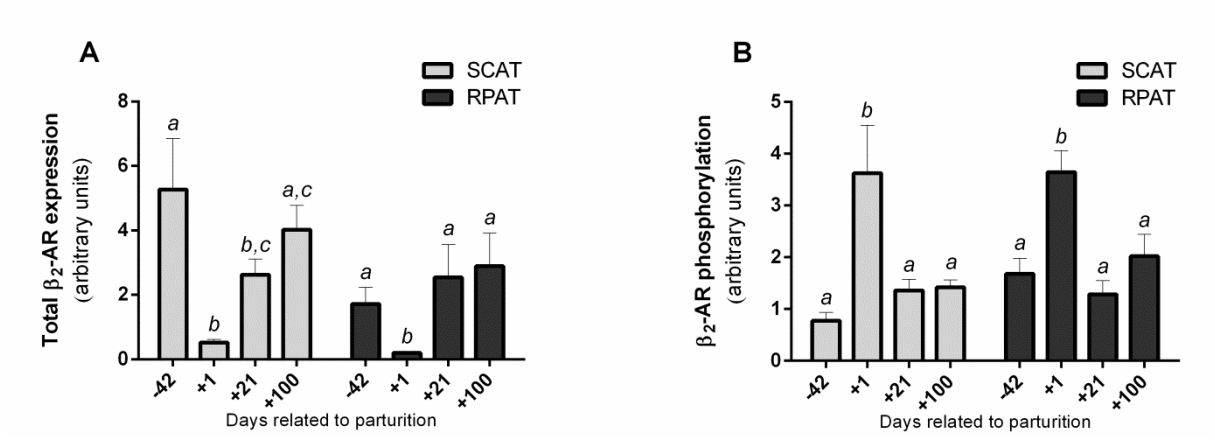

Figure 14. (A) Total expression and **(B)** extent of phosphorylation of β_2-adrenergic receptor (β_2-AR) in fresh, non-incubated samples of subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT) during the periparturient period. Different superscripts indicate $P < 0.05$. Means ± SEM, n = 20.

5.5. Antilipolytic Effect Mediated by the GPR109A-Pathway

5.5.1. Effect of Nicotinic Acid on Lipolysis

Isoproterenol-treated tissues released greater amounts of glycerol and NEFA, and had greater HSL protein phosphorylation than untreated (basal) samples ($P < 0.01$, data not shown). Samples treated simultaneously with isoproterenol and NAC at any concentration showed remarkably lower glycerol release ($P < 0.001$), NEFA release ($P < 0.001$), and HSL phosphorylation ($P < 0.001$) compared to samples treated with isoproterenol only as shown in **Fig. 15**.

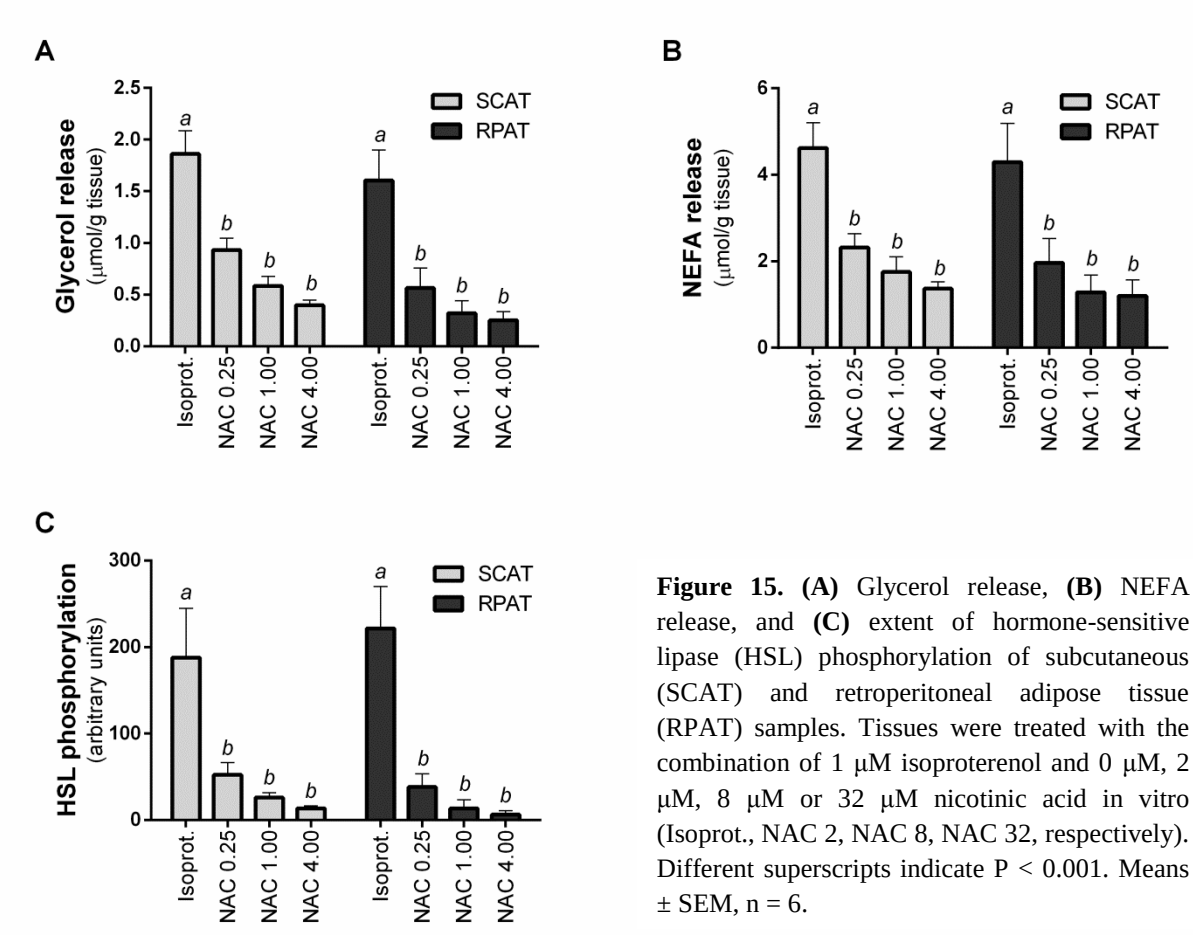

Figure 15. (A) Glycerol release, **(B)** NEFA release, and **(C)** extent of hormone-sensitive lipase (HSL) phosphorylation of subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT) samples. Tissues were treated with the combination of 1 µM isoproterenol and 0 µM, 2 µM, 8 µM or 32 µM nicotinic acid in vitro (Isoprot., NAC 2, NAC 8, NAC 32, respectively). Different superscripts indicate $P < 0.001$. Means ± SEM, n = 6.

5.5.2. Effect of Nicotinamide on Lipolysis

Isoproterenol-treated tissues released greater amounts of glycerol and NEFA, and had greater HSL protein phosphorylation than untreated (basal) samples ($P < 0.01$, data not shown). Samples treated simultaneously with isoproterenol and NAM at any concentration

showed no significant differences in glycerol release, NEFA release, or HSL phosphorylation compared to samples treated with isoproterenol only as shown in **Fig. 16**.

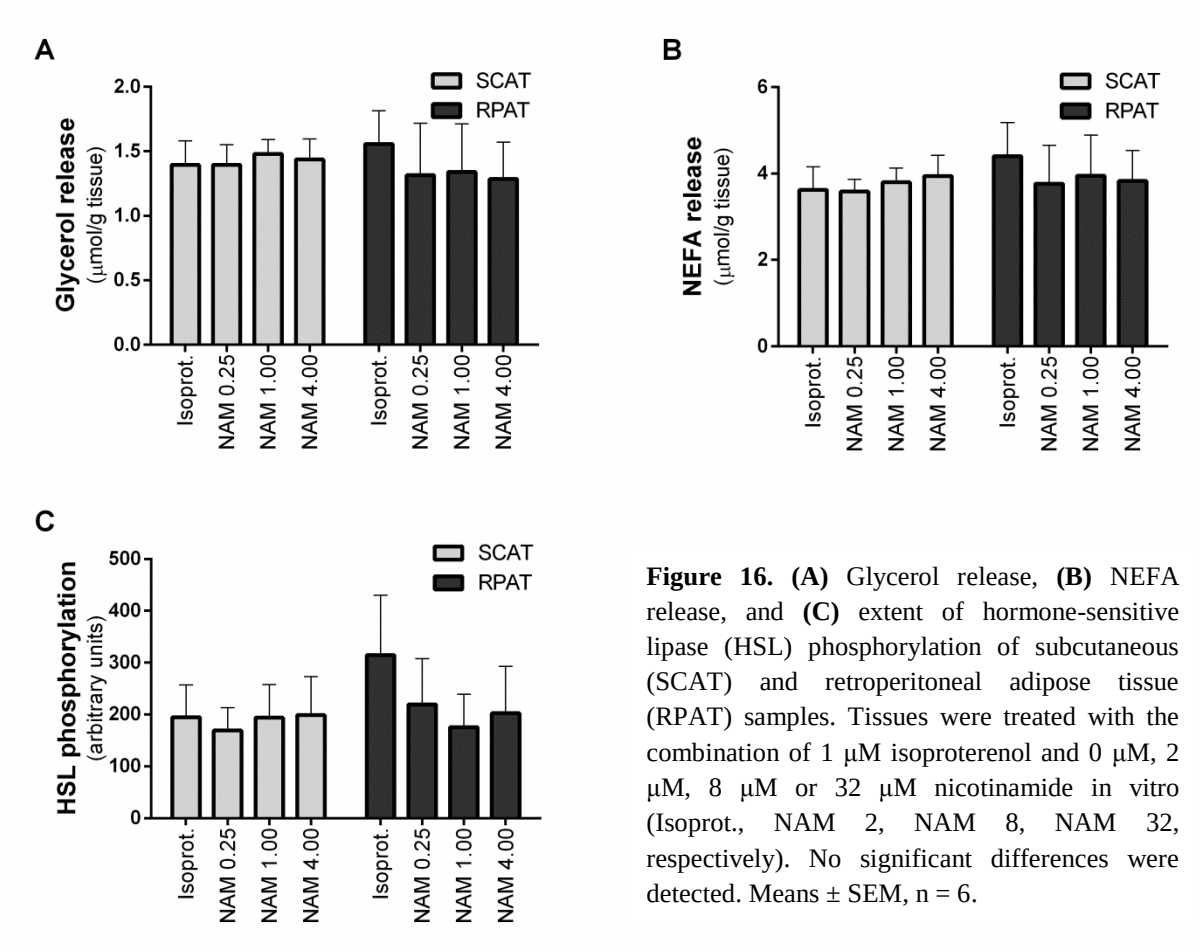

Figure 16. (A) Glycerol release, **(B)** NEFA release, and **(C)** extent of hormone-sensitive lipase (HSL) phosphorylation of subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT) samples. Tissues were treated with the combination of 1 µM isoproterenol and 0 µM, 2 µM, 8 µM or 32 µM nicotinamide in vitro (Isoprot., NAM 2, NAM 8, NAM 32, respectively). No significant differences were detected. Means ± SEM, n = 6.

5.5.3. Expression of the Receptor Protein GPR109A

G protein-coupled receptor 109A protein expression was greater in SCAT than in RPAT ($P < 0.001$) as shown in **Fig. 17**.

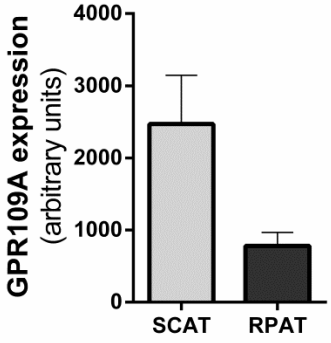

Figure 17. Expression of the receptor protein GPR109A in samples of subcutaneous (SCAT) and retroperitoneal adipose tissue (RPAT). Means ± SEM, n = 10.

6. Discussion

6.1. Animal Production Performance as Influenced by Dietary Interventions

Main finding. Production performance and common metabolic measures changed throughout the periparturient period as expected. However, targeted dietary interventions as high or low energy intake and NAC supplementation did not significantly affect these performance and metabolic changes.

Lack of improvement of the tensed periparturient metabolic condition despite the applied dietary interventions impressively showed that the adaptation to lactation is highly conserved, most likely by genetically and epigenetically driven mechanisms (Loor et al., 2013). Metabolic imbalance, which is a preliminary stage of periparturient production diseases, occurs when this adaptation is disturbed. Certainly there are risk factors such as high milk yield, high body condition at calving, high parity, long dry period associated with the incidence of metabolic diseases (Chen et al., 2015; Vanholder et al., 2015). However, the question why certain cows become diseased, while others in the same herd can cope well with metabolic stress, has still not yet been fully elucidated. The ability of high yielding dairy cows to fulfill the metabolic challenge in the transition period is also discussed to be determined by nutritional management (Roche et al., 2013). The present study was designed to investigate this possibility, i.e. how targeted dietary interventions can influence lipid metabolism and thereby metabolic health.

A high concentrate feeding regimen prepartum was previously showed to increase energy intake (Petzold et al., 2014; Schulz et al., 2014). This could initiate an enhanced anabolic state to develop a bigger mass of adipose depots. Consequently, such overconditioned cows were shown to have greater plasma NEFA concentrations postpartum, associated with a higher incidence of metabolic diseases (Garnsworthy and Topps, 1982; Reid et al., 1986; Treacher et al., 1986; Kokkonen et al., 2005). Nevertheless, it has to be noted that an elevated plasma NEFA level does not necessarily mean higher lipolytic rates, as it could also indicate an inefficient oxidative capacity of liver and muscles, resulting in an accumulation of NEFAs in the circulation (Vernon, 2005).

The extent and timing of lipid mobilization varies greatly due to genetic background, body condition, type and intake of diet and amount of milk production (McNamara, 2012). Based on this previous knowledge, the difference in the energy density and dynamic changes

over time of the LC and the HC diet in the present study were intended to influence lipolytic adaptation in adipose tissues. In particular, the LC diet was adjusted more closely to the actual energy need (i.e. less energy intake before parturition and increasing energy intake faster after parturition), to promote less mobilization. The HC diet was formulated to allow more energy getting stored in the period before parturition, and provide less energy at the onset of lactation, therefore promoting a more intensive mobilization. In contrast, in cows preselected for high BCS, prepartum overconditioning by high energy supply was shown to be associated with higher plasma NEFA levels and more pronounced mobilization postpartum (Garnsworthy and Topps, 1982; Reid et al., 1986; Treacher et al., 1986; Rukkwamsuk et al., 1998; Kokkonen et al., 2005; Janovick and Drackley, 2010; Schulz et al., 2014). In contrast to this, the present study was conducted on cows without preselection for high BCS, receiving a diet with different energy density prepartum to assess the effect of energy intake. Feeding an energy-dense diet prepartum did not affect metabolic performance on the animal level as shown in Fig. 4 and Fig. 5 and further discussed in Tienken et al. (2015, manuscript under revision). On a molecular level, this was also confirmed in the same cows, as markers of lipolysis and lipogenesis in adipose tissues during the periparturient period were not affected by the diet. It is suggested that the selection for high BCS prepartum represents the selection of genetic predisposition for an anabolic metabolic status, in agreement with Kokkonen et al. (2005).

Nicotinic acid is known to have an antilipolytic effect as it was shown already many decades ago to effectively decrease plasma NEFA concentrations in cows (Schultz, 1971; Fronk and Schultz, 1979; Dufva et al., 1983). These early studies observed antilipolytic effects after applying NAC at concentrations of 12, 25 or 40 g/day per cow. In recent years, the antilipolytic effect was confirmed by abomasal infusion at concentrations as low as 6 mg/kg body weight **(BW)**, which corresponds to a single infusion of approx. 4.8 g/cow (Pires and Grummer, 2007) and also by dietary supplementation of rumen-protected NAC at concentrations of 9.6 or 8.0 g/day per cow bioavailable vitamin (Morey et al., 2011; Yuan et al., 2012). A more mechanistic insight into the antilipolytic effect of NAC is provided by the in vitro part of the present study (see chapter *"6.5. Antilipolytic Effect Mediated by the GPR109A-Pathway"*), demonstrating the inhibitory effect of NAC on NEFA and glycerol release and the extent of HSL phosphorylation in dairy cow adipose tissue explants (Kenéz et al., 2014). In the current animal study, the applied dose of 24 g/day per cow dietary non-rumen-protected NAC was intended to suppress lipolysis, especially in the HC group, which was supposed to mobilize more body fat. However, a high proportion of NAC is known to

become destroyed in the rumen (Santschi et al., 2005), and the low amounts of NAC escaping rumen degradation probably did not lead to effective plasma concentrations. After absorption in the gut, a rapid metabolic transformation of NAC into NAM and the coenzyme NAD in the liver could also lead to a loss of antilipolytic action (Niehoff et al., 2009). Consequently, adipose tissues were most likely not exposed to an effective concentration of NAC in the feeding trial of the present study.

6.2. Morphological Adaptation of Adipose Tissues during the Periparturient Period

Main finding. Changes of adipose cell size were in agreement with the emergence of an intensive lipolysis due to the need for energy mobilization during early lactation. However, the tissue composition data, such as TG content, seemed to be contradictory with these changes. Remarkable depot-selective differences were observed between adipose tissues.

The switch from anabolism to catabolism in the transition period requires sufficient adipose tissue plasticity, and has to be reflected by morphological and structural transformation. One general question in this context is whether the periparturient remodeling of adipose tissue occurs mainly by loss of adipocytes during lipid mobilization and proliferation of new adipocytes during the anabolic period, or by the adjustment of adipocyte size, therefore maintaining cell number at a nearly constant level within a depot. In fact, it is technically difficult to follow changes in cell number within an adipose depot throughout the periparturient period. Nevertheless, it was expected that changes in histomorphology and tissue composition can reveal further details on the periparturient remodeling process of adipose tissues.

As a technical note, it has to be mentioned that the analysis of biopsy samples provides information of single time points as snapshots, and it cannot be extrapolated how the studied variables were changing in the time frames between the samplings. However, these sampling times were chosen to give a representative overview of the periparturient period: 42 d prepartum reflected metabolic situation in the dry period with a positive energy balance, at 1 d postpartum lipid mobilization has already begun typically associated with high plasma NEFA concentrations, at 21 d postpartum lipid mobilization still lasts due to ongoing negative energy balance, and at 100 d postpartum cows usually just have reached positive energy balance again (Drackley, 1999; McNamara, 1991; Drackley et al., 2005). Changes in adipocyte size due to lipid mobilization in early lactation have already been observed (Pike

and Roberts, 1984; Akter et al., 2011; Häussler et al., 2013). However, the novelty of the current approach was to link changes of cell size within a tissue with differences in the composition of that tissue. This linkage was provided by examining different aspects of morphology in the exact same tissue samples, and furthermore by extending these observations to both the prepartum and the postpartum period in consecutively obtained tissue samples of the exact same animals. This provides a broader insight into periparturient plasticity of subcutaneous and abdominal adipose tissues, at least at a structural level.

The observed differences in adipose cell size are in agreement with the findings of Akter et al. (2011) and von Soosten et al. (2011) describing a decrease in adipocyte size and depot mass between 1 d and 105 d postpartum with a clear significance in RPAT and in a statistically non-significant manner in SCAT. Furthermore, changes of cell size were in agreement with the well described dynamics of lipid metabolism during the periparturient period. This includes a switch from anabolism to catabolism in adipose tissues associated with a decreased lipogenesis and an increased lipolysis, driven by a negative energy balance during early lactation (Vernon, 1981; McNamara and Hillers, 1986b; Drackley, 1999; Vernon, 2005; Rocco and McNamara, 2013). In the present study, postpartum lipid mobilization was reflected by cell sizes becoming smaller at the postpartum time points, which can be explained by an ongoing depletion of the lipid droplet, resulting in an overall shrinkage of the adipocyte. Accordingly, the decreased number of large cells and the increased number of small cells at 100 d postpartum can be interpreted as a still lasting lipid mobilization. However, it could also be attributed to preadipocyte differentiation and development of new adipocytes. Physiologically, cows are already back to a positive energy balance at 100 d postpartum, which enables the spare energy to be deposited in form of TGs in the adipocytes (Smith and Crouse, 1984; McNamara, 1994; Hausman et al., 2009). This TG deposition was most likely associated with differentiation of preadipocytes leading to the formation of early adipocytes (Spalding et al., 2008; Hausman et al., 2009; Albrecht et al., 2015). This assumption is supported by the findings of Häussler et al. (2013), observing a significantly greater percentage of preadipocytes at 42 d postpartum than at 105 d postpartum in bovine RPAT, indicating that many preadipocytes differentiated in this time span.

Furthermore, it is not known if adipocytes within an adipose depot have a uniform metabolic capacity, or if groups of cells respond with a different extent to lipolytic and lipogenic stimuli, resulting in a heterogeneous metabolic pattern of the depot. This could be possible due to non-homogenous receptor distribution between cells deriving from different preadipocyte subpopulations, or different amounts of hormones reaching the cells because of

uneven density of capillarization. The consequence of this would be that cells which respond to a lesser extent to lipolytic stimuli remain in the depot while others might become depleted and undergo apoptosis. Assuming that depot size shrinkage happened mainly by loss of adipocytes in times of a negative energy balance, it is proposed that the greater amount of small cells observed at 100 d postpartum represented a newly differentiating population of adipocytes which grew besides the remained "poor responder" cells.

Tissue composition analysis was performed to gain more information about adipose tissue restructuring in addition to cell size changes. As the DNA content of a single cell is known to be relatively constant, it was intended to be used as an indicator of cell number (Adams and Storrie, 1981). The observed greater DNA content at 100 d postpartum suggests that one gram of tissue contained a higher number of cells, which is in accordance with the small cell sizes registered in the histomorphometric analysis of RPAT. However, the question still remains open if these small cells are existing adipocytes with decreased TG content or newly proliferating adipocytes with fresh TG filling. Not even the TG content analysis gives an answer to this question, as this remained at an equal level throughout all the 4 investigated time points. A possible explanation for this might be that, compared with all other components, TGs have a paramount proportion in adipose tissue. Accordingly, even if the amount of TG within the cells declines, one gram of tissue (even containing varying number of cells) could maintain constant levels of TG content. Therefore, fewer large cells filled with much TG or more small cells containing little TG could still result in approximately the same TG content per gram tissue, because all other cell components generally add up only a minor fraction. Nevertheless, a remarkable depot-specific difference was revealed by the TG content analysis, as RPAT contained much less TG than SCAT. Also, RPAT was found to have greater total protein content and less cytoskeletal β-actin protein expression than SCAT at similar cell size values, which together suggests that RPAT is less limited to be a tissue serving only as a passive storage for fats. Accordingly, the lower proportion of cytoskeletal structure (β-actin) and the higher proportion of total protein (enzymes, receptors, cell organelles etc.) suggest greater metabolic activity for RPAT as an abdominal adipose depot. This is in agreement with Locher et al. (2011, 2012), also concluding an enhanced metabolic flexibility of RPAT based on greater hormone-sensitive lipase and AMP-activated protein kinase phosphorylation rates in this depot in periparturient dairy cows. Furthermore, these findings are in agreement with Akter et al. (2011) and von Soosten et al. (2011) as well, observing a more prominent decrease of RPAT depot mass and RPAT adipocyte size compared to SCAT during the first 105 d of lactation.

Previous research revealed that dairy cows can mobilize up to 0.6 kg/day body fat during early lactation (Tamminga et al., 1997). In this context, one could expect even more remarkable differences in the studied histomorphometric and composition variables between the postpartum time points. On the contrary, SCAT DNA content, SCAT and RPAT TG content, SCAT protein content, and SCAT and RPAT β-actin expression were not significantly different between the investigated postpartum time points. This observation suggests that the depots might be indeed heterogeneous in their metabolic pattern comprising adipocytes that respond to a greater extent to lipolytic stimuli and therefore disappear quickly, and adipocytes which are "poor responders" and therefore remain in the depot. Taking adipose biopsy samples postpartum which contain many of these remaining cells could be an explanation for the lack of differences in the mentioned variables.

6.3. Extent of Lipolysis and Lipogenesis during the Periparturient Period

Main finding. Phosphorylation of HSL and perilipin was increased, while FAS protein expression was decreased during early lactation both in SCAT and in RPAT, irrespectively of high or low prepartum dietary energy density and dietary NAC supplementation.

Adipose tissue lipolysis in cows is known to be regulated primarily at a posttranslational level via catecholamine stimulation (as reviewed in McNamara, 2012) by promoting HSL and perilipin phosphorylation (Sumner and McNamara, 2007; Elkins and Spurlock, 2009; Koltes and Spurlock, 2011; Locher et al., 2011; Rocco and McNamara, 2013). Therefore, the extent of phosphorylation of HSL and perilipin at specific amino acid residues are considered as relevant indicators of current lipolytic activity (also see chapter 6.4.). Furthermore, previous research has shown correlations between HSL activity with milk production, consistent with a connection through endocrine and neuroendocrine systems (McNamara, 1989; Sumner and McNamara, 2007).

Phosphorylation sites and their physiological meaning have been heavily studied in various species, and among several amino acid residues of HSL, Ser563 (according to the rat sequence) was identified as the major activator regulatory site (Yeaman, 2004). This phosphorylation site corresponds to Ser552 of the bovine HSL sequence, and has been confirmed to be an important regulatory target of lipolysis in periparturient dairy cows (Kenéz et al., 2014). On the other hand, less is known about the role of perilipin phosphorylation in bovine adipose tissues. The phosphorylation of perilipin A (the major isoform in adipocytes)

at Ser522 (according to the human sequence) was found to be the most important protein kinase A-dependent target in mouse adipocytes (the homologous site is Ser517 in the murine, and Ser516 in the bovine protein sequence) (Miyoshi et al., 2007). In previous studies, the total phosphorylation of perilipin has already been used as an indicator of lipolysis in bovine adipose tissue (Elkins and Spurlock, 2009; Koltes and Spurlock, 2011; Faylon et al., 2014).

The current work extends our understanding about the contribution of HSL and perilipin phosphorylation to the intensive catecholamine-driven lipolysis occurring during the transition period in cows. In agreement with previous work, the present study demonstrated that phosphorylation of HSL and perilipin in bovine SCAT and RPAT was already increased shortly after parturition, whereas the total abundance of these proteins remained nearly constant throughout early and mid lactation (Koltes and Spurlock, 2011; Locher et al., 2011). Also the transcriptional production of mRNA for these proteins was previously shown to increase during the postpartum period; however, this did not occur until later in lactation and was highly related to the amount of milk production, not just the lactational state (Sumner and McNamara, 2007). Therefore, it must be reminded that the total metabolic flux in adipocytes is a function of many processes such as energy availability, energy demand by the mammary gland and other tissues and the balance of lipolytic and lipogenic pathways.

The transition period is associated with a marked reduction in transcription of genes coding for lipogenic proteins and in lipogenic activity of adipose tissue (Sumner and McNamara, 2007; Khan et al., 2013; Rocco and McNamara, 2013). In some animals, lipogenesis may rebound from its nadir within 3 to 4 weeks of lactation, while in others the period of reduced anabolism can extend several weeks (McNamara, 1994; Rocco and McNamara, 2013). As reflected by the present results on FAS protein expression, lipogenesis was clearly downregulated during early lactation as a consequence of the onset of a negative energetic state. As energy intake and net energy balance was increasing at 100 d postpartum, fatty acid synthesis in adipose tissues was already upregulated again, even though phosphorylation of HSL and perilipin was also still high due to the peak milk production. It is clear from this work and past that in fact lipogenesis is mainly a function of energy intake, whereas the phosphorylation of HSL and perilipin is directly related to the activity of the mammary gland as suggested many years ago (Vernon, 1981) and shown in various studies since (reviewed in McNamara, 2012). It is likely that the latter is a direct effect of increased sympathetic nervous system activity during lactation (McNamara and Murray, 2001).

In previous studies, a greater metabolic flexibility of RPAT over SCAT was suggested (Locher et al., 2011, 2012), and other abdominal adipose tissues of cows were reported to

respond to overfeeding by becoming remarkably increased in mass, without any significant increase of BCS (Drackley et al., 2014). However, the present study revealed only minor differences between SCAT and RPAT regarding lipolysis and lipogenesis, and therefore the assumption that abdominal adipose tissues provide more NEFA due to greater lipolytic potential, as suggested by a more intensive mobilization of the RPAT depot (von Soosten et al., 2011), was not confirmed. The commonly used measure of BCS and the associated risk assessment for metabolic disorders in dairy management is based on the evaluation of the visible SCAT depots only. Therefore, depot-specific differences are an important aspect in the risk assessment for periparturient metabolic disturbances. Differences between adipose depots are interesting from a quantitative biology point of view as well, provided the possibility that subcutaneous and abdominal adipose masses still contribute to the overall energy supply of the animal at different rates. To clarify this issue and to get a more precise view of energy homeorhesis at an organism level, further studies on metabolic activity, particularly energy efflux rates from various adipose depots are warranted.

In agreement with the absence of dietary effects on animal production data (see chapter 6.1.), markers of lipolysis and lipogenesis were not influenced by dietary energy intake or NAC supplementation either. The HC group was hypothesized to have a more intensive lipolysis than the LC group, reflected by greater HSL and perilipin phosphorylation. Furthermore, the NAC supplemented group was hypothesized to have a restricted lipolysis due to the antilipolytic effect mediated by the GPR109A-pathway. The absence of these effects suggests that the activation of the cellular lipolytic pathway is not highly sensitive to dietary factors. Most likely, variation in lipolytic activity can rather be attributed to the actual energy demand of milk synthesis. This energy demand could be influenced mainly by the stage of lactation and by genetic merit of the cow, as suggested by Vernon (1981) and McNamara (2012).

6.4. β-Adrenergic Response of Adipose Tissues during the Periparturient Period

Main finding. Adipose tissue explants responded with increased NEFA and glycerol release and concurrently with increased HSL and perilipin phosphorylation to isoproterenol stimulation in vitro. The applied β-adrenergic stimulus triggered the highest response at 42 d prepartum. Postpartum when in vivo lipolysis was upregulated, tissue explants had a lower response to the same defined isoproterenol stimulus.

To extend our understanding of lipolytic control, an in vitro assay was used in the current study to monitor β-adrenergic response of adipose tissue explants and to track how far this is in consistence with the lipolytic status in vivo. As measures of lipolytic response given to a defined β-adrenergic stimulus, NEFA and glycerol release and HSL and perilipin phosphorylation of adipose tissue explants were used. Also the causal link between protein phosphorylation and the functional metabolic response was confirmed by the strong correlation observed between these measures. This also extends previous work revealing correlations between plasma NEFA and glycerol concentrations and HSL and perilipin phosphorylation levels in non-incubated bovine SCAT during early lactation (Elkins and Spurlock, 2009).

The applied concentration of isoproterenol (1 μM) allowed registering the maximal capacity of lipolysis (lipolytic responsiveness under maximal stimulation). This consideration is based on previous work investigating the effect of different isoproterenol concentrations (Smith and McNamara, 1989; Khan et al., 2013). Unexpectedly, the applied β-adrenergic stimulus triggered the highest response at 42 d prepartum, and in contrast to the initial hypothesis, postpartum tissue explants had a lower response to the same defined stimulus. The decrease of β-adrenergic response from 42 d prepartum to 1 d postpartum is certainly affected by decreased receptor protein expression and concurrently increased receptor phosphorylation. It should to be reminded that in contrast to HSL and perilipin, the phosphorylation of the β-adrenergic receptor causes inactivation. This is a form of agonist-mediated receptor desensitization, as the activated lipolytic cascade is known to exert a negative feedback effect on the β-receptors (Hausdorff et al., 1990; Lefkowitz et al., 1990). This negative feedback loop affecting the β-receptors can be promoted by an increased adrenergic stimulus, which in fact occurs in the cow during the days around calving due to an increase in sympathetic tone (McNamara, 1991). At 21 and 100 d postpartum the receptor protein expression was increasing again. Earlier studies showed that β-adrenergic receptor binding capacity increased during lactation (Jaster and Wegner, 1981), and also the mRNA for β_2-adrenergic receptors, similarly to HSL and perilipin mRNA, was shown to increase postpartum in direct relation to rates of milk production (Sumner and McNamara, 2007). Besides the regulation through expression and desensitization of the β-adrenergic receptors, further regulatory mechanisms have to be considered to explain the modest lipolytic response. Prepartum, antilipolytic factors such as insulin, α_2 or adenosine receptor signaling are most likely important for suppressing lipolysis and restraining HSL and perilipin phosphorylation at a low level, therefore the increased lipolysis seen during early lactation might also be a

result of a deterioration of these antilipolytic effects. In addition, the significance of several other pro-lipolytic endocrine factors such as glucocorticoids, glucagon and growth hormone has to be taken into consideration as well. The contribution of the mentioned factors to lipolytic adaptation in dairy cows should be elucidated in further studies.

Of all the proteins involved in the adipose lipolytic pathway, HSL was recognized as the rate-limiting key enzyme for TG hydrolysis for a long time. In agreement with this, the present study confirmed that Ser563 in bovine HSL protein is an important phosphorylation site inducing an increase in NEFA and glycerol release and thus possessing a central regulatory function in lipolysis. Isoproterenol as a well-known activator of the β-adrenergic pathway increased lipolysis as described in previous studies (McNamara et al., 1992; Khan et al., 2013). The mechanism of this action incorporates the β-adrenoceptor – cAMP – protein kinase A – HSL axis, leading to the phosphorylation of HSL (Holm et al., 2000; Sumner and McNamara, 2007). However, recent studies revealed that the involvement of various lipases in TG hydrolysis is altered by different metabolic conditions (reviewed in Kraemer and Shen, 2006; Lass et al., 2011). Also in the current study, basal incubation conditions were associated with remarkably low HSL phosphorylation throughout the whole test period. Now it is known that in fact basal or constitutive lipolysis is catalyzed by a different enzyme, adipose triglyceride lipase **(ATGL)** which is not activated with phosphorylation (Miyoshi et al., 2008; Elkins and Spurlock, 2010; Koltes and Spurlock, 2011). Treating the tissue samples with 1 μM isoproterenol triggered an approx. 15-20-times greater extent of HSL phosphorylation than basal incubation. Thus there are two points of control, basal lipolysis by ATGL action, and β-adrenergic stimulated lipolysis by HSL action. This finding helps explain the different pattern of adaptation of basal and stimulated lipolysis in the current study and also in previous work (McNamara and Hillers, 1986a; Khan et al., 2013; Rocco and McNamara, 2013). Furthermore, the remarkably lower phosphorylation of HSL under basal incubation conditions compared to the non-incubated tissues shows that phosphorylation could be effectively diminished within an incubation assay of 90 min. Accordingly, phosphorylation and dephosphorylation of HSL was confirmed to be a quick regulatory mechanism in response to a changing stimulatory environment. In contrast to HSL, perilipin phosphorylation was not that remarkably reduced during basal incubation, but rather maintained at a moderate level. This also indicates that lipolysis can be supported even in the absence of β-adrenergic stimuli; particularly, through mechanisms controlled by phosphorylation of perilipin, such as increasing accessibility to the lipid droplet and activating ATGL by releasing comparative

gene identity-58 (CGI-58) (Yamaguchi et al., 2006; Granneman et al., 2009; Koltes and Spurlock, 2011).

6.5. Antilipolytic Effect Mediated by the GPR109A-Pathway

Main finding. The GPR109A-mediated antilipolytic pathway does exist in a functioning form in bovine SCAT and RPAT. The stimulation of this pathway with the receptor agonist NAC, but not with NAM, resulted in a decreased lipolytic response of adipose tissues under in vitro conditions, which was mediated by a reduced phosphorylation of HSL.

In this trial, the causal role of the GPR109A-mediated antilipolytic pathway in the lipolytic fine-tuning machinery of bovine adipose tissues was examined. To stimulate these pathways, ex vivo treatments of tissue samples with NAC and NAM were performed using concentrations which can physiologically occur in the blood of dairy cows (Campbell et al., 1994; Niehoff et al., 2009). Also in this study, both SCAT and RPAT were examined, because GPR109A was shown to differ in the level of its protein expression between different adipose tissues in Holstein steers (Titgemeyer et al., 2011). According to our results, GPR109A protein expression also varied between SCAT and RPAT of dairy cows. However, different levels of receptor protein abundance did not result in a different antilipolytic response. In spite of a 3-fold greater receptor protein expression in SCAT, the inhibitory effect of NAC on glycerol and NEFA release and HSL phosphorylation was not enhanced compared to RPAT. This may be explained by adjustments through constitutive counteracting pathways or differences in receptor-ligand affinity.

Nicotinic acid decreased HSL phosphorylation at Ser563 and consequently reduced the lipolytic response. This indicates that the GPR109A-mediated pathway described in murine and human adipocytes (Tunaru et al., 2003) is present in dairy cow adipose tissues as well and is an efficient way to downregulate lipolysis, at least in vitro. In the context of lipid mobilization in the dairy cow, this in vitro finding marks a potential control point to restrict NEFA release from adipose depots in a physiological way via the GPR109A-mediated pathway. Supporting the plasma NEFA-reducing effect of in vivo NAC treatment demonstrated by Morey et al. (2011) and Titgemeyer et al. (2011), the present in vitro results suggest that an enhanced plasma concentration of NAC could cause a restricted level of HSL phosphorylation, possibly preventing excessive rates of lipolysis. The dietary relevance of this

antilipolytic mechanism was tested in our feeding trial (described in chapter 4.1.2.) by supplementing NAC to the cows and subsequently analyzing phosphorylation of HSL and perilipin as cellular markers of lipolysis. As described in chapter 5.1, dietary supplementation of 24 g/d per cow non-rumen-protected NAC did not significantly affect HSL and perilipin phosphorylation in vivo. The reason for this could be extensive rumen degradation or a rapid hepatic bioconversion resulting in insufficient plasma concentrations of NAC reaching the adipose tissues.

Although NAM does not differ very much in its biochemical properties from NAC, results of the present study clearly showed that NAM was not capable of suppressing lipolytic activity in isolated bovine adipose tissues. Previous reports regarding the antilipolytic effects of NAM were somewhat inconsistent. In a feeding trial, NAM supplementation of lactating dairy cows was associated with decreased plasma NEFA and BHBA concentrations, indicating reduced lipid mobilization (Jaster and Ward, 1990). On the other hand, studies investigating receptor binding revealed that NAM was inactive on GPR109A (Lorenzen et al., 2001; Wise, 2003). In rat epididymal adipose tissue explants, comparing antilipolytic effects of NAC and NAM also demonstrated that NAC inhibited catecholamine-induced lipolysis, but NAM did not (Carlson, 1963). To sum up, these observations suggest that, unlike NAC, NAM cannot exert any inhibiting effect on catecholamine-induced lipolysis because it cannot activate the GPR109A-mediated pathway.

Besides NAC, also BHBA acts as an agonist of GPR109A, albeit with a 8,000-times less activity (Taggart et al., 2005). As reported in Kenéz et al. (2014), in vitro treatment with pathophysiologically high (ketotic) concentrations of BHBA could also decrease glycerol release and HSL phosphorylation in bovine adipose tissues. Therefore, the relevance of this metabolic pathway lays not only in downregulating lipolysis by dietary factors, but also by intrinsic factors, i.e. by an elevated plasma BHBA concentration. This could act as a negative feedback mechanism to restrain lipolysis in ketosis.

In view of a broader scope of control of lipolysis, the present study provides evidence about the existence and functionality of the GPR109A-mediated pathway as a mechanism to downregulate lipolysis in subcutaneous and visceral adipose tissues in cows. In previous research approaches, a number of cellular mechanisms were found to have antilipolytic effects in bovine adipose tissue, including α_2-adrenergic receptor signaling (Smith and McNamara, 1989), AMP-activated protein kinase signaling (Garton et al., 1989) and A_1-adenosine receptor signaling (Houseknecht et al., 1995). Nonetheless, these antilipolytic pathways are difficult to be influenced by exogenous factors such as dietary supplements. This underlines

the relevance of the GPR109A-mediated pathway as this might serve as a dietary accessible control point (e.g. by NAC supplementation) to restrict excessive lipolysis and contribute to a balanced lipid metabolism in periparturient dairy cows. In fact, dietary nicotinic acid supplementation is not new to dairy nutrition, as previous in vivo studies found reduced plasma NEFA concentrations after NAC administration (for a review see Niehoff et al., 2009). Nevertheless, plasma NEFA concentrations are always determined by multicausal effects modulating NEFA release and utilization. In this respect, the current tissue explant study demonstrating the antilipolytic effect of NAC via the GPR109A-mediated pathway confirms and extends the previous in vivo findings.

7. Conclusions

Metabolic changes of the transition period induced extensive adaptation processes in subcutaneous and abdominal adipose tissues, which could be observed at morphological, molecular and functional levels as well. These observations contribute to a better understanding of regulatory mechanism controlling adipose tissue metabolism in dairy cows in the periparturient period, and therefore can be used to develop strategies aiming the improvement of metabolic health in dairy farming.

Generally, it is desirable to manage energy homeostasis and maintain metabolic health via dietary interventions. An optimal body condition at calving is considered to be crucial for minimizing the risk of postpartum metabolic disorders; however, the current findings suggest that an altered dietary energy intake during the dry period might not be sufficient to significantly change postpartum lipid metabolism in cows with a homogenous body condition. Therefore, postpartum lipid metabolism might rather be affected by high or low body condition reflecting a higher or lower capability for anabolism or catabolism determined by genetic traits.

Nicotinic acid, but not NAM was shown in vitro to reduce lipolysis when applied in physiological concentrations. The lack of an antilipolytic effect after dietary non-rumen-protected NAC supplementation suggests that feeding an encapsulated form could possibly be beneficial to achieve an antilipolytic effect in vivo, due to improved bioavailability for absorption in the gut. However, a bioconversion of the absorbed NAC in the liver could have accounted for a rapid hepatic clearance, so that adipose tissues could not be reached by sufficient amounts to activate the GPR109A-pathway. In this respect, it cannot be excluded that the well-described plasma NEFA-lowering effect is based on improving hepatic oxidative capacity by NAC, rather than affecting adipose tissue metabolism. Nevertheless, further studies are necessary to confirm or reject this speculation.

The current data on lipolysis and in vitro β-adrenergic response confirm that post-translational activation of HSL and perilipin via specific phosphorylation sites is a major control mechanism of lipid flux in the response to lactation. To cover the energy need of lactation and maintenance, lipolysis becomes upregulated concurrently to the onset of lactation and is maintained at least up to 100 d postpartum. Lipogenesis is much more a function of energy intake, accordingly the synthesis of fatty acids has its nadir shortly after parturition and as net energy balance increases, lipogenesis becomes active again. Nevertheless, lipolysis and lipogenesis are both embedded into a complex metabolic network

affected by anabolic and catabolic factors, that is why further research and data interpretation is warranted to understand interactions between HSL and perilipin controlled lipolysis, lipolysis via other lipases (such as ATGL), NEFA reesterification and lipogenesis under different aspects of energy homeostasis.

Findings of the morphological investigations suggest that adipose tissue depots with an ongoing lipolysis shrunk mainly by reducing the number of adipocyte within a depot. The possibility of a uniform decrease of all adipocytes in their size throughout the whole depot was not supported by the results. It seems more likely that certain mature adipocyte subpopulations of the depot became depleted and destructed in association with the need for energy mobilization, while others remained less affected by metabolic signals. This might be explained by non-homogenous receptor distribution or capillarization throughout the adipose tissue depots. Further immunohistochemical studies are warranted to confirm this. Based on the comparison between depots, RPAT is suggested to undergo a more dynamical remodeling process during the periparturient period, implying a higher plasticity of RPAT compared to SCAT.

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