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Cellular energy supply and aging in dairy cows:

Characterization of different physiological states and impact of diet-induced over-condition

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English abstract

Lactation in dairy cows is accompanied by dramatic changes in energy balance and thus requires the continued adaption of the key organs, namely adipose tissue (AT), liver, and mammary gland to the varying conditions. The supply of energy by mitochondria, the "powerhouses" of the cell, therefore is of pivotal importance in dairy cows, because both the number of the mitochondria and the copy number of their own genome, the mitochondrial DNA (mtDNA), can change according to different physiological, physical and environmental stimuli. Moreover, determination of the length of telomeres, short repetitive DNA sequences at the end of chromosomes, has become a common method in human research to determine an individual's physiological age. Due to the fact, that telomeres shorten with every cell division and this shortening is influenced by diet, metabolic stress, and diseases, telomere length (TL) in dairy cows might serve as a phenotypic biomarker for longevity. The aim of this dissertation was to characterize the effects of lactation and the influences of a 15-weeks period of diet-induced over-condition on mitochondrial biogenesis, variation of TL and on markers for oxidative stress in dairy cows. Furthermore, as lipogenic and lipolytic processes during lactation result in changes of AT mass, we aimed to investigate angiogenesis and hypoxia in AT after an excessive fat accumulation. The mtDNA content and TL in blood as well as in AT, mammary gland, and liver of primiparous (PP) and multiparous (MP) dairy cows were studied during early and late lactation. Furthermore, the expression of genes related to mitochondrial biogenesis was measured in tissue samples of these cows as well as in AT of over-conditioned, non-lactating dairy cows. The effects of over-condition on oxidative stress related changes in mtDNA content in non-lactating cows were also examined. From early to late lactation, tissue mtDNA copy numbers increased in all lactating cows in a tissue-specific manner, whereas blood mtDNA content decreased during this period. The highest mtDNA content found in liver emphasizes the crucial metabolic role of this organ in dairy cows. Also mRNA expression of mitochondrial biogenesis related genes changed tissue-dependently, whereby the transcriptional regulation of mtDNA was limited to AT. Strong correlations between blood and tissue mtDNA during early lactation were observed, suggesting blood mtDNA measurements for indirectly assessing the energy status of tissues and thus substituting tissue biopsies. Telomeres were only shortened in blood and mammary gland from early to late lactation and the rate of shortening was dependent on the initial TL in all investigated samples. Due to diet-induced over-condition, the markers for oxidative stress increased in non-lactating cows, which might in turn impair mtDNA. Furthermore, enlarged adipocytes showed signs of hypoxia, indicating insufficient angiogenesis in AT. The ascending mtDNA content might improve the energy supply and thus compensate the hypoxic condition in rapidly expanding AT. The results in the present dissertation provide a longitudinal characterization of mtDNA content and mitochondrial biogenesis as well as TL in different tissues and in blood from dairy cows during lactation. Therefore, this thesis serves as a basis for further studies elucidating the role and regulation of mitochondria and telomeres in various pathophysiological conditions in cattle.

German abstract

Die Laktation von Hochleistungskühen wird begleitet von beträchtlichen Veränderungen in der Energiebilanz der Tiere. Die hauptsächlich an der Laktation beteiligten Organe, Fettgewebe, Leber und Milchdrüse müssen sich daher kontinuierlich an die variierenden Bedingungen anpassen. Mitochondrien, die „Kraftwerke" der Zellen, sorgen für eine ausgewogene Energieversorgung und sind daher ein wichtiger Bestandteil im Organismus von Milchkühen. Die Mitochondrienanzahl sowie die Kopienzahl des mitochondrialen Genoms, die mitochondriale DNA (mtDNA), kann sich entsprechend physiologischer, organischer und umweltbedingter Stimuli verändern. In den Humanwissenschaften ist die Bestimmung der Telomerlängen (TL) eine gebräuchliche Methode, um das physiologische Alter eines Individuums zu definieren. Telomere sind kurze, sich wiederholende DNA-Sequenzen an den Chromosomenenden, die sich mit jeder Zellteilung verkürzen. Zusätzlich wird die TL-Verkürzung durch Ernährung, metabolischen Stress und Erkrankungen beeinflusst. Demnach könnte die Bestimmung der TL auch in Milchkühen als Biomarker für die genetische Selektion auf Langlebigkeit von Bedeutung sein. Ziel dieser Dissertation ist es, den Einfluss der Laktation und die Auswirkung einer 15-wöchigen fütterungsbedingten Überkonditionierung auf die mitochondriale Biogenese, die TL und auf Marker von oxidativem Stress in hochleistenden Milchkühen zu charakterisieren. Der mtDNA-Gehalt und die TL im Blut sowie im Fettgewebe, Leber und Milchdrüse wurde bei primiparen (PP) und multiparen (MP) Milchkühen während der Früh- und Spätlaktation untersucht. Die Expression von Genen der mitochondrialen Biogenese wurde ebenfalls in den Gewebeproben dieser Tiere ermittelt, sowie im Fettgewebe von überkonditionierten, nicht-laktierenden Milchkühen. Da die während der Laktation ablaufende Lipogenese und Lipolyse Veränderungen in der Fettgewebsmasse verursachen, war ein weiteres Ziel dieser Arbeit, die Untersuchung der Angiogenese und Hypoxie im Fettgewebe nach einer exzessiven Fettanreicherung. Zusätzlich wurden die Auswirkungen einer Überkonditionierung auf die aus oxidativem Stress resultierenden Veränderungen des mtDNA-Gehaltes im Fettgewebe von nicht-laktierenden Kühen erforscht. Die mtDNA Kopienzahl in den überprüften Geweben hat sich von der Früh-zur Spätlaktation bei allen laktierenden Kühen gewebsspezifisch erhöht, während sich der mtDNA-Gehalt des Blutes in diesem Zeitraum reduzierte. Die essenzielle metabolische Rolle der Leber bei Milchkühen spiegelt sich durch den dort beobachteten höchsten mtDNA-Gehalt wider. Die mRNA Expression von mitochondrialen Genen war ebenso wie die mtDNA gewebsspezifisch verändert, wobei eine Regulation der mtDNA auf transkriptioneller Ebene nur im Fettgewebe eine Rolle zu spielen scheint. Aufgrund einer starken Korrelation zwischen dem mtDNA-Gehalt im Blut und dem in Geweben während der Frühlaktation, könnte die Messung der mtDNA im Blut ein potentielles Medium sein um den Energiestatus von Geweben widerzuspiegeln und Gewebebiopsien zu substituieren. Die TL haben sich nur im Blut und der Milchdrüse von der Früh- zur Spätlaktation verkürzt, wobei das Ausmaß der Reduktion in allen untersuchten Proben abhängig von den Ausgangs-TL war. Nicht-laktierende Milchkühe zeigten bei der fütterungsinduzierten Überkonditionierung erhöhte Konzentrationen an Indikatoren für oxidativen Stress, welche zu Schäden der mtDNA führen können. Des Weiteren wurde festgestellt, dass eine Vergrößerung der Adipozyten mit einer Hypoxie einherging, welche auf eine unzureichende Angiogenese im Fettgewebe hinweist. Daher lässt sich mutmaßen, dass ein Anstieg des mtDNA-Gehaltes die Energieversorgung in dem sich schnell vergrößernden Fettgewebe verbessert und damit die Hypoxie kompensiert werden kann. Die Ergebnisse der vorliegenden Dissertation zeigen die Veränderungen des mtDNA-Gehaltes, der mitochondrialen Biogenese sowie der TL in verschiedenen Geweben und Blut von Milchkühen währen der Laktation. Somit dient diese Arbeit als Grundlage für weitere Untersuchungen, um die Rolle und Regulation von Mitochondrien und Telomeren in verschiedenen pathophysiologischen Stadien von Kühen zu erforschen.

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1   Introduction

Milk production of dairy cows is increasing steadily; modern high-yielding Holstein Friesian cows can produce around 55 kg milk per day (Breves, 2007). Genetic selection for increased productivity can have negative side effects on animal health and welfare. Reduced fertility, lameness, metabolic disorders, compromised immune function and thus increased susceptibility towards infectious diseases are just a few examples being responsible for the continuously shortened productive life of the animals (Sordillo et al., 2009).

Reducing these negative effects with the objective to combine high performance and health requires a profound knowledge of the cow's physiology.

1.1   The physiological states of lactation in high-yielding dairy cows

The metabolic situation of dairy cows passes different stages during lactation, caused by variations in milk production as well as changes in feed intake and body condition. Thereby critical times, characterized by dramatic changes in energy balance and metabolic status, are shortly before calving (3 wk ante partum) and in early post partum (3 wk post partum), taken together as the so-called transition period (Grummer, 1995). The transition period determines the productivity and thus the profitability of dairy cows, as health disorders, nutrient deficiency or poor management can inhibit their ability to reach maximal performance (Drackley, 1999). Metabolic, physical and hormonal changes around calving result in a decline of voluntary feed intake (Allen et al., 2005). Consequently, the consumed feed alone cannot compensate the high energy demands for the increased milk production and thus results in a negative energy balance (NEB). In order to meet the elevated energy needs for lactation, cows mobilize body reserves mainly from adipose tissue (AT) to support maintenance and milk production. During fat mobilization, also referred to as lipolysis, triglycerides stored in AT are hydrolyzed into glycerol and free fatty acids, which are released into the circulation as non-esterified fatty acids (NEFA). In mid- and late lactation voluntary feed intake is high enough to compensate for the loss of energy with milk; moreover, milk synthesis starts to decrease and thus the energy required for milk production is less; however, energy is still important for pregnancy and restoring body reserves for the next lactation. The AT depots are refilled due to fat accumulation (lipogenesis) during mid and late lactation and the beginning of the dry period when animals are in a state of positive energy balance.

1.1.1   Metabolic and oxidative status in over-conditioned dairy cows

The rate and extent of AT mobilization depend on several factors including body condition score (BCS) at calving, composition of the diet, milk production and parity (Komaragiri et al., 1998). Transition cows with high BCS lose more body condition and body weight than thinner cows (Treacher et al., 1986). At the onset of lactation, over-conditioned cows [BCS > 4; Edmonson et al., (1989)] are disposed to rapid and excessive lipolysis; their NEFA concentrations released into the bloodstream are higher as compared to cows with moderate or low BCS (Pires et al., 2013). Thus, over-conditioned cows are susceptible to develop metabolic disorders as well as health and reproduction problems and are especially sensitive to oxidative stress (Morrow et al., 1979; Gearhart et al. 1990; Dechow et al., 2004; Bernabucci et al., 2005). Hyperlipidemia leads to reduced insulin sensitivity of peripheral tissues (Bell, 1995; Holtenius et al., 2003; Hayirli, 2006) and can result in insulin resistance in dairy cows (Pires et al., 2007). The uptake of high amounts of NEFA from the liver may result in an increased risk for the fatty liver syndrome, when triglyceride synthesis exceeds the hepatic export capacity (Bobe et al., 2004), and influences neutrophil function (Scalia et al., 2006). Furthermore, excessive fat mobilization leads to elevated circulating concentrations of β-hydroxybutyrate (BHB). High concentrations of BHB and NEFA in turn are associated with a higher incidence of ketosis and also with compromised immune functions (Drackley, 1999; Herdt, 2000).

Oxidative stress describes the imbalance between the production of reactive oxygen metabolites (ROM) and antioxidant defense mechanisms, in which ROM exceed the neutralizing capacity of antioxidants. A certain amount of reactive oxygen species (ROS), mainly derived by mitochondria, is desirable, as ROS can increase the oxygenation of other molecules involved in the regulation of important cellular functions such as differentiation and proliferation (Halliwell and Gutteridege, 2007). However, overproduction of ROS that cannot be counterbalanced by antioxidants can damage all major classes of biomolecules, and lead to pathological changes (Lykkesfeldt and Svendsen, 2007) and reproductive problems in dairy cows (Miller et al., 1993).
In humans, oxidative stress is associated with obesity and insulin resistance (Higdon and Frei, 2003; Keaney et al., 2003). Similarly, in dairy cows oxidative status may change depending on the metabolic status (Bernabucci et al., 2005). In the study quoted above, dairy cows with a high BCS at calving and a greater BCS loss after calving had increased levels of oxidative stress post

partum. Furthermore, oxidative stress in transition dairy cows contributes to various disorders such as milk fever, mastitis and impaired reproductive performance (Miller et al., 1993).

### 1.1.2 The importance of adipose tissue in dairy cows

The AT plays a central role in homeostatic and metabolic regulation, not only because of its ability to store and mobilize triglycerides, but also because of its function as an endocrine, autocrine and paracrine gland. It is a type of loose connective tissue composed of adipocytes, collagen fibers and cells belonging to the so-called stromal vascular fraction such as preadipocytes, endothelial cells, fibroblasts, blood vessels, immune cells and nerves (Frayn et al., 2003). The AT is highly vascularized, and each adipocyte is provided with an extensive capillary network (Silverman et al., 1988). The secretion of numerous bioactive molecules, namely adipokines (e.g. adiponectin, leptin, resistin, visfatin, apelin) allows AT to communicate with the liver, muscles, brain, reproductive- and other organs of the body. Furthermore, adipokines and thus AT are involved in various physiological and metabolic processes such as lipid,- glucose,- and energy metabolism, appetite regulation, vascular homeostasis, insulin sensitivity, inflammation and immune function (Frühbeck, 2008).

Depending on the cellular structure and functions, AT can be classified in two main types: brown AT (BAT) and white AT (WAT). The regulation of thermogenesis is the main function of BAT, which consists of several small lipid droplets and a distinctly high number of mitochondria (Tran and Kahn, 2010). The most abundant type of AT in adults is WAT that is characterized by adipocytes containing a single lipid droplet, an eccentrically located nucleus and a relatively small number of mitochondria at the cell periphery (Shen et al., 2003). The WAT is the AT type in focus of this thesis.

1.1.3 Adipose tissue angiogenesis

During lipogenesis, the mass of WAT can increase via hypertrophy of adipocytes or increase its cell number by hyperplasia, or by combinations of these two processes, whereas during lipolysis adipocytes reduce their volume (hypotrophy). To fulfill these dynamic processes, as well as to provide sufficient oxygen and nutrients for the cells and/or to support NEFA and glycerol release, WAT requires continuous remodeling of its vascular network via angiogenesis (Lu et al., 2012; Elias et al., 2013; Lemoine et al., 2013). Thus, the ability of AT to adapt to varying energy demands depends mainly on the vasculature (Rupnick et al., 2002).

The processes of angiogenesis and vasculogenesis are closely connected, but execute different functions. Vasculogenesis describes the formation of new blood vessels by assembly of endothelial cells or angioblasts, whereas angiogenesis includes the sprouting and elongation of pre-existing vessels (Risau, 1997; Figure 1).

A   Vasculogenesis

Progenitor cells/ angioblasts

Blood vessels

B   Angiogenesis

C   Vasculogenesis & Angiogenesis
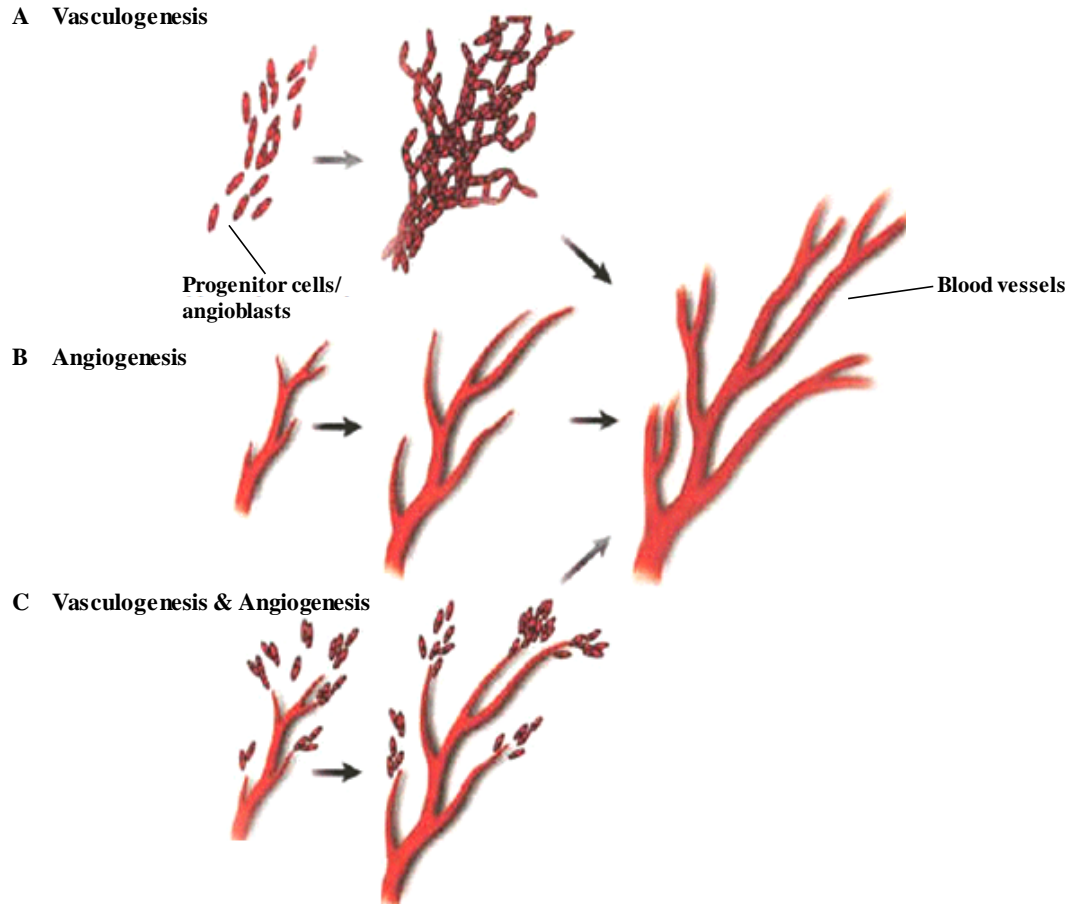
Figure 1: Schematic representation of angiogenesis and vasculogenesis. (A) Vasculogenesis is the development of blood vessels by conflating angioblasts or endothelial progenitor cells. (B) Angiogenesis is the formation of new blood vessels by sprouting and elongation of pre-existing ones. It includes the proliferation and migration of differentiated endothelial cells. (C) Angiogenesis and vasculogenesis can also occur at the same time. Modified according to Cleaver and Krieg (1998).

The key regulator of blood vessel growth and remodeling is the vascular endothelial growth factor A [VEGF-A or VEGF; (Tam et al., 2009)]. The VEGF promotes and stimulates development, proliferation and permeability of endothelial cells and is regarded as a survival factor *in vivo* and *in vitro* by preventing endothelial cells from apoptosis (Ferrara and Alitalo, 1999; Shibuya, 2001). The mitogenic, angiogenic and permeability-enhancing effects of VEGF

are mainly mediated through the tyrosine kinase receptor VEGF-R2, located on the cell surface (Terman et al., 1991; Shalaby et al., 1995).

The expansion of AT during lipogenesis leads to an increase in the intercapillary distance of hypertrophied adipocytes, resulting in decreased blood flow of the tissue and consequently reduced oxygen supply. Insufficient oxygen supply of a tissue leads to local hypoxia, which in turn, contributes to angiogenesis by inducing a number of growth factors. In obese humans and mice, for example, the hypoxia-inducible-factor 1α (HIF- 1α), the major marker for hypoxia in AT, is upregulated and therefore initiates expression of VEGF (Scannell et al., 1995; Mason et al., 2007; Lemoine et al., 2013). Furthermore, hypoxia has been associated with AT dysfunction (Hosogai et al., 2007), inflammation (Ye et al., 2007) and cell death (Yin et al., 2009).

1.2   Cellular energy-supply in metabolism of dairy cows

Energy consumption after calving dramatically increases to support the onset of milk synthesis and secretion. Nutrients, such as glucose, amino acids, fatty acids and molecular oxygen are used as energy sources which are required to fuel proper physiological functions. These multiple metabolic reactions are collectively referred to as cellular respiration. It is one of the key pathways of cells to gain useable energy to fulfill cellular activity. The chemical energy stored in form of adenosine triphosphate (ATP) can be used to drive energy-dependent processes, including biosynthesis or transportation of molecules across cell membranes. The generation of ATP by glycolysis, mainly derives from processes taking place in mitochondria, the powerhouses of the cell.

1.2.1   The role of mitochondria in cellular metabolism

Mitochondria are double-membrane organelles and the major components of energy metabolism in most mammalian cells. They contribute to essential cellular processes, which are merged and interdependently forming a complex network.

Mitochondria are located in all cell types except red blood cells (Stier et al., 2013). Their number, size and shape are tissue- and cell-type specific and dependent on the metabolic activity and thus on the energy requirements of the cell (Fawcett, 1981). A brain cell may have around 2000 mitochondria (Uranova et al., 2001), whereas a white blood cell exhibits less than a hundred

(Selak et al., 2011) and a hepatocyte may have between 800 and 2000 mitochondria (Fawcett, 1981).

The most important processes for ATP generation are through electron transport and oxidative phosphorylation (OXPHOS), in combination with the catabolism of fatty acids via β-oxidation and oxidation of metabolites by the tricarboxylic acid (TCA) cycle. These reactions are performed by components of the respiratory chain (RC) located in the inner mitochondrial membrane (Lee et al., 2000). A byproduct of the RC is the production of ROS. Mitochondria control the ability of cells to generate and detoxify ROS, but they also represent an immediate target of ROS (Nicholls et al., 2003).

In addition to the production of energy, mitochondria participate in activating apoptosis (programmed cell death), through the release of mitochondrial proteins into the cytoplasm.

Mitochondria possess their own genome, the mitochondrial DNA (mtDNA) located in the mitochondrial matrix. The mitochondria genome encodes 37 genes: 22 tRNAs, a small (12S) and a large (16S) rRNA and 13 polypeptides encoding subunits of the electron transport chain including ATP synthase (Wallace, 1994). Transcription, translation and replication of mtDNA are implemented within the mitochondria; however, most of the enzymes and proteins that are located in the mitochondrial membrane are nuclear gene products, with their main function to synthesize ATP (Lee et al., 2000). Furthermore, these nuclear encoded proteins influence proliferation, localization and metabolism of mitochondria (Lopez et al., 2000; Calvo et al., 2006).

1.2.2   Mitochondrial DNA copy number

The mtDNA is a circular, double-stranded molecule (Wallace, 1994) that exists with 2-10 copies in each mitochondrion of mammalian cells (Robin and Wong, 1988). The mtDNA content per mitochondrion in a given cell type, between cells from different mammalian tissues and between different species is essentially constant (Bogenhagen and Clayton, 1974; Robin and Wong, 1988). The copy number of mtDNA is thus a marker of mitochondrial proliferation and reflects the abundance of mitochondria in a cell (Izquierdo et al., 1995).

Unlike the nuclear DNA (nDNA), the mtDNA is unmethylated, lacks introns and is not protected by histones (Groot and Kroon, 1979). Owing to its lack of histones and the close proximity of

mtDNA to production sites of ROS by the RC, mtDNA is susceptible to oxidative damages by ROS attack (Ide et al., 2001; Santos et al., 2003).

The mtDNA copy number in human cells differs among the types of cells and tissues (Robin and Wong, 1988; Renis et al., 1989; Falkenberg et al., 2007) and can be modified according to the energy demands of the cell and under varying physiological or environmental conditions (Lee and Wei, 2005).

Variations of the mtDNA copy number were found to be associated with oxidative stress, obesity and aging in numerous human cells and tissues, including skeletal muscle (Barrientos et al., 1997a), brain (Barrientos et al., 1997b), leukocytes (Liu et al., 2003) and AT (Choo et al., 2006; Rong et al., 2007). Increased copy numbers of mtDNA might act as a compensatory mechanism to oxidative DNA damage, mtDNA mutations and decline in respiratory function; processes that occur during human aging and during conditions of high oxidative stress (Lee et al., 2000).
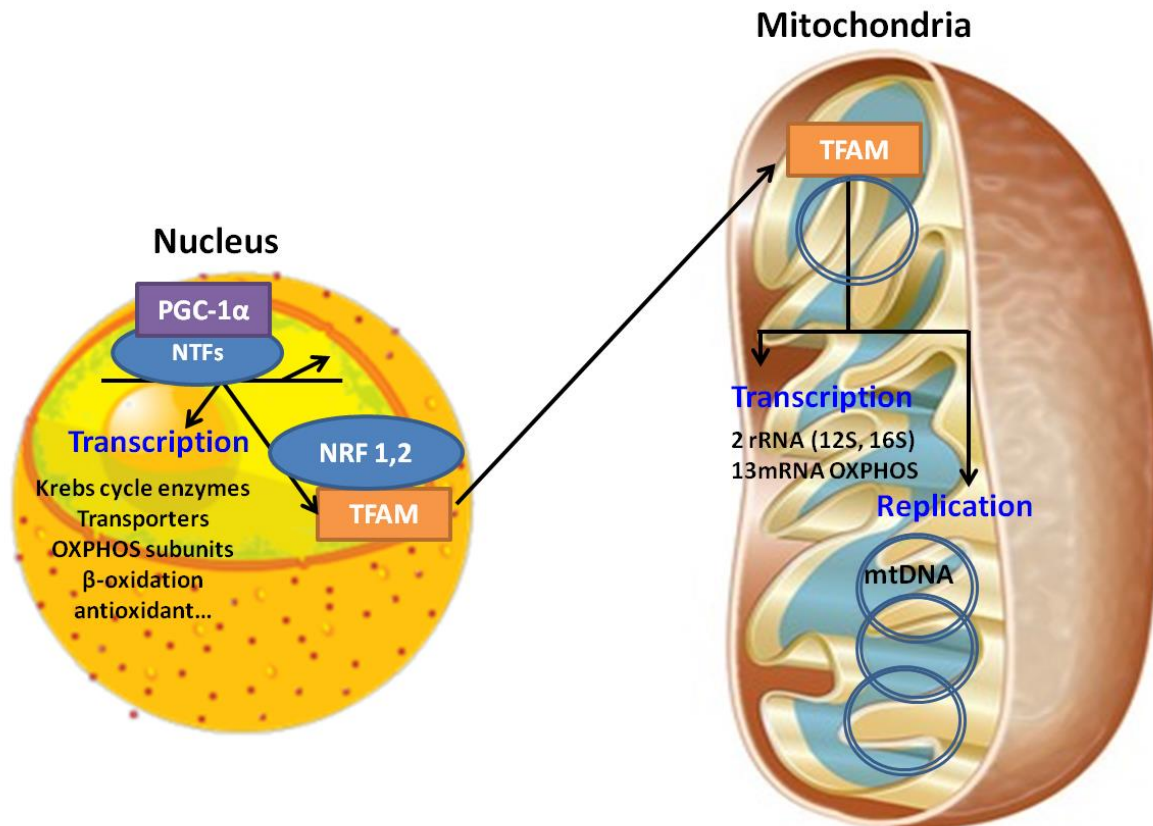
1.2.3   Regulators of mitochondrial biogenesis

Considering the main function of mitochondria, the generation of ATP, mitochondrial biogenesis increases with energy requirements and decreases with energy excess to support the cell under regular conditions and during metabolic stress (Piantadosi and Suliman, 2012).

Mitochondrial biogenesis includes both mitochondrial proliferation and differentiation events (Izquierdo et al., 1995).

The replication of mtDNA occurs independently of nuclear DNA replication (Bogenhagen and Clayton, 1977). However, most of the proteins and enzymes involved in regulation of mitochondrial gene expression are encoded by nuclear genes (Scarpulla, 1997; Shadel and Clayton, 1997), also called transcription factors.

The major regulators for the replication and transcription of the mitochondrial genome include the mitochondrial transcription factor A (TFAM), RNA polymerase (POLRMT), DNA polymerase (POLG), nuclear respiratory factor 1 (NRF-1), GA-binding protein-α [GABPA or nuclear respiratory factor 2 (NRF-2)] and peroxisome proliferator-activated receptor gamma coactivator 1-alpha [(PGC-1α); Malik and Czajka, 2013]. Their mRNAs are translated in the cytoplasm and the proteins are imported into the mitochondria (Figure 2).

Figure 2 Simplified schematic representation of mitochondrial biogenesis. Peroxisome proliferator-activated receptor gamma co-activator 1-alpha (PGC-1α) activates nuclear transcription factors (NTFs) leading to transcription of nuclear-encoded proteins and of the mitochondrial transcription factor A (TFAM). The TFAM promoter contains recognition sites for nuclear respiratory factors 1 and/or 2 (NRF-1,-2), thus allowing coordination between mitochondrial and nuclear activation during mitochondrial biogenesis. TFAM activates transcription and replication of the mitochondrial genome. OXPHOS: oxidative phosphorylation. Modified according to Ventura-Clapier et al. (2008).

TFAM participates in the initiation and regulation of mtDNA transcription and replication (Virbasius and Scarpulla, 1994; Larsson et al., 1998). This major transcription factor is able to pack and unwind mtDNA (Fisher et al., 1992) and is indispensable for mtDNA maintenance as a main component of the mitochondrial nucleoid (Kang et al., 2007). Variations in the amount of mtDNA occur concomitantly with variations in the amount of TFAM in human cells, underlining the key role of TFAM in mtDNA copy number regulation (Poulton et al., 1994; Shadel and Clayton, 1997).

The expression of TFAM is regulated by nuclear transcription factors. Therefore, TFAM exhibits *inter alia* binding-sites for NRF-1 and NRF-2 (Virbasius et al., 1993; Virbasius and Scarpulla, 1994). These factors coordinate the gene expression between the mitochondria and the nuclear

genome by transmitting nuclear regulatory events via TFAM to the mitochondria (Virbasius and Scarpulla, 1994; Gugneja et al., 1995).

PGC-1α stimulates the expression of NRF-1 and NRF-2 and is integrated in the expression of genes of the aerobic metabolism. In transgenic mice, overexpression of PGC-1α leads to mitochondrial proliferation in adipocytes (Lowell and Spiegelman, 2000) and heart (Lehman et al., 2000), assuming a key role for PGC-1α in the control of mtDNA maintenance.

1.2.4   Mitochondria in dairy cattle

To our knowledge, there is no report in the literature about the mtDNA copy number and the molecular mechanisms responsible for the replication and transcriptional activation of mtDNA during lactation in dairy cattle. Its amount in key organs related to lactation, such as AT, liver and mammary gland has also not been discussed yet. However, a few studies describe mtDNA variations during bovine embryogenesis *in vitro*. For example, mtDNA copy numbers were higher in bovine embryos at the blastocyst stage compared to mouse embryos (Smith et al., 2005), indicating that DNA replication in the bovine species occurs during early embryogenesis. Furthermore, mtDNA copy numbers in bovine oocytes were 100-fold higher compared to somatic cells (bovine fetal heart fibroblasts), underlining a vital role for mtDNA during bovine oogenesis. It was also noted that genotypic differences in the amount of mtDNA between individual oocytes from the same animal might occur in cattle (Michaels et al., 1982). May-Panloup et al. (2005) emphasized the importance of mitochondrial biogenesis activators such as TFAM and NRF-1 for bovine embryogenesis, as they found high levels of both factors from the bovine oocyte stage onwards.

1.3   Processes of cellular aging

The term "aging" defines the progressive functional reduction of tissue capacity that may lead to mortality, resulting from a decrease or a loss of function of postmitotic cells to maintain replication and cell divisions (Kirkwood and Holliday, 1979).

Many theories of aging have been proposed, whereby modern biological theories can be divided in two main categories: programmed theories and damage or error theories. The programmed theories are based on the assumption that aging follows a biological timetable, in which

regulation depends on gene expression changes that affect the systems responsible for maintenance, repair and defense mechanisms. According to the damage or error theories, aging is a consequence of environmental assaults to organisms that induce cumulative damage at various levels (Jin, 2010).

Biological aging and DNA damage are strictly connected, as there are numerous examples in the literature illustrating an age-related decline in DNA repair capacity.

Enhanced oxidative damage, reduced DNA repair capacity and resulting mutations, altered signaling that impairs tissue response to injury or disease, and changes in global or specific gene expression patterns are just a few examples of the broad cellular processes and changes associated with aging (Lee et al., 1998a).

Chromosomes become increasingly damaged with age (Hastie et al., 1990). Telomeres cap the end of chromosomes, giving them stability and a protection against degradation (Blackburn, 2001). Telomeres normally counteract age-dependent damage, however when they fail the protective function, the standard cellular response that activates the DNA-repair machinery is triggered. This response, which involves the protein p53, stops DNA replication and other cellular proliferative processes. If repair fails, the cell may undergo apoptotic cell death.

The telomere shortening theory of aging is a widely accepted mechanism, as telomeres have been shown to shorten with each successive cell division. Shortened telomeres activate p53, which in turn prevents further cell proliferation and triggers cell death (Lee et al., 1998b).

In addition, mitochondria are suggested to play a role in aging; as it is proposed, that mutations progressively accumulate within the mtDNA that is nearly unprotected, leading to energetic deficient cells (Balaban et al., 2005; Wallace, 2005). Furthermore, it has also been implied that the activity of master regulators (e.g. PGC-1α) of mitochondrial biogenesis decreases with aging leading to mitochondrial dysfunction. Age-dependent variations in the number of mitochondria are controversially discussed. Decline in the amount and function of mtDNA triggered by decreased PGC-1α expression may give rise to enhanced ROS production (Finley and Haigis, 2009); however, enhanced ROS concentration may increase the amount of mtDNA to compensate mitochondrial damage in elderly subjects (Ames et al., 1995; Figure 3).

This study will rather focus on the roles of telomeres in cell aging than on age-dependent mitochondrial dysfunctions.
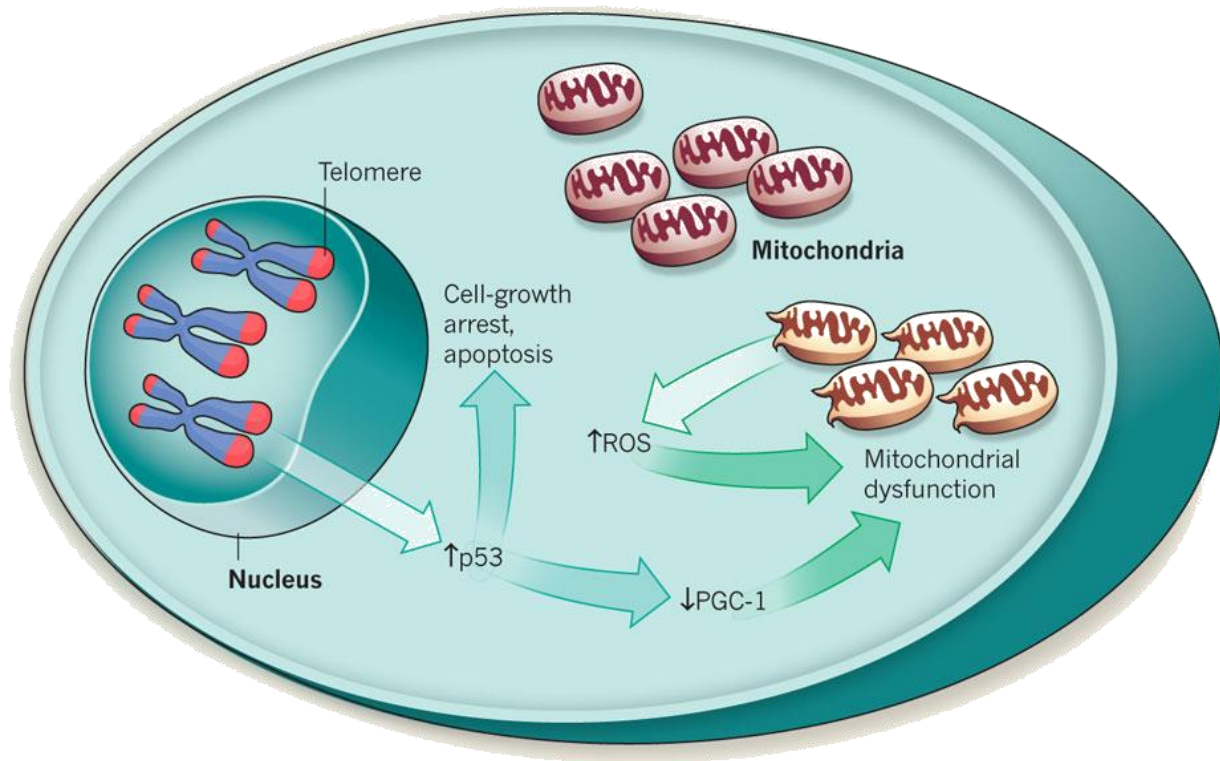
Figure 3 With age, shortened, defective telomeres will trigger DNA damage signals such as p53, which can have multiple effects. Proliferative cells respond by inhibition of DNA replication and cell growth leading to either apoptosis or senescence. Age-related dysfunctions of mitochondria in quiescent tissues also result from p53 activity by repression of PGC-1 and concomitant reduction of mitochondria numbers and functions. Dysfunctional mitochondria in turn, will enhance generation of reactive oxygen species (ROS) which result in further mitochondrial DNA damage. From Kelly (2011).

1.3.1   Telomeres and the end-replication problem

The proliferative capacity of normal cells is limited, referred to as the "Hayflick limit" (Hayflick, 1965; Campisi, 1997) and controlled by a cellular generational clock (Shay et al., 1991).

Telomeres are repetitive DNA sequences (TTAGGG) at the end of chromosomes (Zakian, 1989) that ensure chromosome stability and protect against degradation and fusion (Blackburn, 2001). Loss of telomeres results from the "end-replication problem", the inability of DNA polymerase to entirely replicate the end of DNA strands. The shortening of telomeres is associated with normal aging in all somatic tissues and with cell divisions, leading to genomic instability (Zakian, 1989; Harley et al., 1990; Counter et al., 1992).

The reverse transcriptase enzyme telomerase maintains and elongates telomere length (TL) by adding TTAGGG repeats to telomeres and thus allows cells to overcome cellular senescence (Shay and Bacchetti, 1997; Autexier and Lue, 2006; Collins, 2006). Telomeres can switch from

an "open" state, allowing elongation by telomerase, to a "closed" state with inaccessibility to telomerase and vice versa (Blackburn, 2001; Figure 4). It has been indicated, that the likelihood of the open state is proportional to the TL of the repeat tracts (Surralles et al., 1999). In most somatic cells addition of telomeric repeats by telomerase is outbalanced by repeat losses.
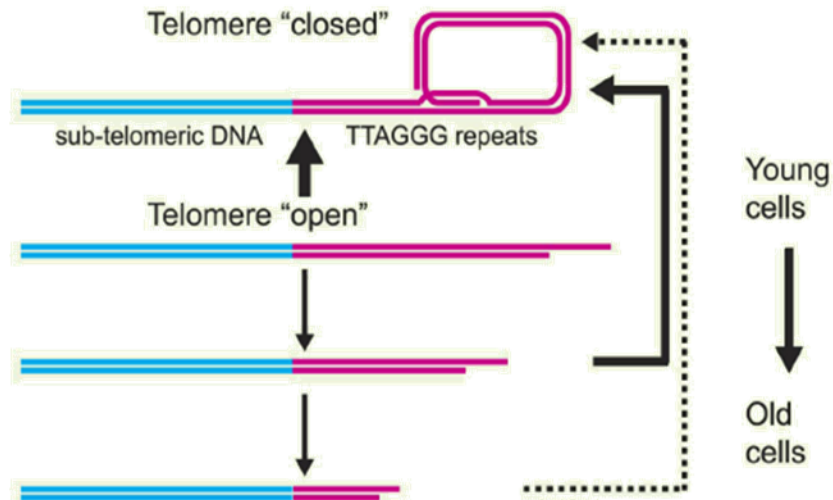
Figure 4 Telomeres in young cells have long tracts of telomeric repeats (TTAGGG repeats) that favor folding into a "closed" structure that is inaccessible to telomerase and DNA damage response pathways. As the telomere length at individual chromosome ends decreases, the likelihood that telomeres remain "closed" also decreases. At one point telomeres become too short and indistinguishable from broken ends. Depending on the cell type and the genes that are expressed in the cell, a limited number of short ends can be elongated by telomerase or recombination. Continued cell divisions and telomere loss will lead to accumulation of too many short ends. At this point, defective telomeres will trigger DNA damage signals. Modified from Aubert and Lansdorp (2008).

The relative TL varies considerably between species and between individuals of the same age (Ehrlenbach et al., 2009), because TL is influenced by an individual's genetics and environment (Kappei and Londono-Vallejo, 2008). Telomeres play a central role in the cellular response to stress and DNA damage and variations in TL in humans have been related to diet (Marcon et al., 2012), psychological stress (Epel et al., 2004), disease (Jiang et al., 2007) and, naturally, age (Ehrlenbach et al., 2009).

1.3.2   Telomere length in dairy cattle

Only a few studies deal with the topic of telomeres and TL shortening in cattle. Leukocyte TL in Japanese Black cattle have been estimated to vary between 19.0-21.9 kb in calves and 15.1-16.8

kb in 18-year-old animals and are shorter in cloned animals (Miyashita et al., 2002). Brown et al. (2012) recently demonstrated an association of TL shortening with age and herd management in lactating Holstein cows. Furthermore, they concluded that TL might be an indicator of the survival time of dairy cows: cows with short telomeres showed a reduced survival period. In another study of Tilesi et al. (2010), TL variations were found to be related to cattle breeds. The authors of this study compared TL of two beef cattle breeds (Maremmana and Chianina) in liver, lung and spleen tissue and found the longest telomeres in liver. The breed-specific differences in TL were attributed to potential effects arising from crossbreeding.

2   Objectives

Mitochondria are the main sources for energy in cells; however, information about their abundance and gene expression in blood and tissues of dairy cows during different physiological states such as the transition period and late lactation were lacking. In addition, the effect of a diet-induced over-condition, as it might happen in late lactation, on mitochondrial biogenesis and angiogenesis of AT have not been assessed in dairy cattle so far. Furthermore, cell aging, in terms of the investigation of the length of telomeres in dairy cows and potential specific differences in physiologically relevant tissues, such as the liver, mammary gland, and AT of PP and MP cows has not been studied previously. Therefore, the present study was designed to fill these gaps of knowledge with the following objectives:

1) To investigate the effects of a diet-induced over-condition in non-lactating cows on oxidative stress and its impact on mitochondrial biogenesis and angiogenesis in AT,

2) To characterize mtDNA content and mitochondrial biogenesis in blood and in tissues during different stages of lactation in PP and MP dairy cows, and

3) To give an overview about TL and TL- shortening in dairy cows during different stages of lactation.

3   Manuscript I (submitted)

The impact of oxidative stress on adipose tissue angiogenesis and mitochondrial biogenesis in over-conditioned dairy cows

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HIGHLIGHTS

- Diet-induced over-conditioning leads to oxidative stress in non-lactating cows
- The mtDNA copy number increases in adipose tissue during over-conditioning
- Angiogenesis fails to adapt to expanding adipocyte size, leading to hypoxia in AT
- Increased mtDNA content might compensate hypoxic conditions
- Oxidative stress increases mtDNA content without changing mitochondrial biogenesis

ABSTRACT

With the onset of lactation, dairy cows with a BCS > 3.5 are sensitive to oxidative stress and metabolic disorders. Adipose tissue (AT) is able to adapt to varying metabolic demands and energy requirements by the plasticity of its size during lactation. Within AT, angiogenesis is necessary to guarantee sufficient oxygen and nutrient supply for adipocytes. The cellular energy metabolism is mainly reflected by mitochondria, which can be quantified by the mtDNA copy number per cell. In the present study, we aimed to investigate the impact of over-condition on angiogenesis and mitochondrial biogenesis in AT of non-lactating cows, irrespective of the physiological influences of lactation. Therefore, 8 non-pregnant, non-lactating cows received a ration with increasing energy density for a period of 15 weeks during which body weight and body condition were substantially increased. Subcutaneous AT was biopsied every 8 week and blood was sampled monthly. The concentrations of indicators for oxidative stress in blood continuously increased within the experimental period, which might damage mtDNA. Concomitantly HIF-1α, the major marker for hypoxia, increased until experimental week 8, indicating insufficient angiogenesis in the rapidly expanding AT. Based on the observation that the number of apoptotic cells decreased with increasing hypoxia, the detected ascending mtDNA copy numbers might compensate the hypoxic situation within AT, reinforcing the production of oxidative stressors. Key transcription factors of mitochondrial biogenesis were largely unaffected, thus increased oxidative stress will not impair mtDNA.

Keywords: Adipose tissue, Dairy cow, Hypoxia, Mitochondrial Biogenesis, Oxidative Stress

INTRODUCTION

After calving, most cows undergo a phase of negative energy balance (EB), in which the energy demand for milk synthesis cannot be covered by voluntary feed intake. In order to meet the increased energy demands, cows mobilize body reserves predominantly from adipose tissue (AT). In the course of lactation, milk synthesis decreases and the energy depots are refilled leading to a positive EB (Drackley et al., 2005). Over-conditioned cows mobilize more body reserves than thin cows (Treacher et al., 1986) and are more susceptible to metabolic disorders as well as health and reproduction problems (Gearhart et al., 1990; Goff and Horst, 1997; Roche et al., 2009).

During lactation, AT actively adapts to the metabolic needs via mobilization of the energy stores (lipolysis) and refilling of the fat depots (lipogenesis). In obese species the blood supply in AT is adapted to dynamic cellular processes via angiogenesis, in order to provide sufficient nutrients and oxygen for the cells and/or to support the NEFA and glycerol release (Elias et al., 2013; Lemoine et al., 2013; Lu et al., 2012). The vascular endothelial growth factor A (VEGF-A or VEGF) is the key regulator of vasculogenesis and angiogenesis (Tam et al., 2009), stimulating migration, permeability, proliferation and survival of endothelial cells (Ferrara and Alitalo, 1999; Shibuya, 2001). The angiogenic and mitogenic effects of VEGF are mainly mediated through the tyrosine kinase receptor VEGF-R2 (Shalaby et al., 1995; Terman et al., 1991). Within AT, VEGF is suggested to be involved in energy metabolism (Lu et al., 2012) and its increased expression protects against the negative consequences of diet-induced obesity and metabolic dysfunction (Elias et al., 2013).

Rapid expansion of AT and adipocyte sizes leads to an increase of the intercapillary distance, resulting in decreased blood flow and reduced oxygen supply. In obese humans and mice, insufficient oxygen supply of a tissue might cause local hypoxia. In response to hypoxia, AT produces the transcription factor hypoxia-inducible-factor-1α (HIF-1α) which in turn induces angiogenic growth factors (Lemoine et al., 2013; Mason et al., 2007; Scannell et al., 1995). Moreover, up-regulation of HIF-1α can lead to inflammation (Ye et al., 2007) and cell death in AT (Yin et al., 2009).

In cows with a BCS above 3.5 prior to calving and great BCS loss after calving, metabolic stress is accompanied by increased oxidative stress (Bernabucci et al., 2005). Oxidative stress mainly derives from an imbalance between the production of reactive oxygen species (ROS) by mitochondria and antioxidant defenses that convert ROS to less malign molecules (Bernabucci et al., 2005; Sies, 1991). High concentrations of ROS during increased metabolic demands can damage proteins, lipids, DNA as well as mitochondria themselves (Sawyer and Colucci, 2000; Williams, 2000). Mitochondrial DNA (mtDNA) is more susceptible to damages caused by oxidative stress than nuclear DNA (Clayton, 1984). Damaged mtDNA can result in a decline of mtRNA transcription and further lead to dysfunction of mitochondrial biogenesis (Wallace, 1999).

Mitochondrial biogenesis describes both proliferation and differentiation of mitochondria (Izquierdo et al., 1995). One of the main markers of mitochondrial proliferation is the mtDNA copy number per cell (Al-Kafaji and Golbahar, 2013). Genes involved in the transcription, regulation and maintenance of mtDNA, such as the nuclear-respiratory factor 1 and 2 (NRF1, NRF2), mitochondrial transcription factor A (TFAM) and the peroxisome proliferator-activated receptor-γ coactivator (PGC-1α; Izquierdo et al., 1995) may change their expression through varying energy supply (Lee et al., 2008).

In the present study, we hypothesized that over-condition of cows leads to local hypoxia in AT due to insufficient angiogenesis. This might change the cellular energy supply and consequently alter the number of mtDNA copies and/or result in programmed cell death (apoptosis) in AT. Furthermore, oxidative stress might impair the amount and function of mitochondria in bovine AT. In order to describe the local hypoxia and its relation to angiogenesis, we evaluated HIF-1α and the pro-angiogenic factors VEGF-A and VEGF-R2. Moreover, we determined the mtDNA copy number per cell and measured the abundance of genes being involved in the transcription, regulation and maintenance of mtDNA in subcutaneous (sc) AT from over-conditioned cows. In addition, we assessed the concentrations of advanced oxidation protein products (AOPP), of lipid peroxidation via measuring thiobarbituric acid reactive substances (TBARS) and of derivates of reactive oxygen metabolites (dROM) as indicators for oxidative stress and examined their relationship on mtDNA content and mitochondrial biogenesis.

MATERIAL AND METHODS

Experimental setup and sample collection

The animal experiment was performed according to the European Community regulations and admitted by the Lower Saxony State Office for Consumer Protection and Food Safety (LAVES), Germany. The experimental design has been published previously (Dänicke et al., 2014). In brief, eight non-lactating, non-pregnant German Holstein cows (Age: 4 – 6 years) were kept in an open barn and fed solely with straw offered *ad libitium* for 5 months. After this period, i.e., the onset of the present observation period, the portion of straw was gradually decreased and the animals were adapted to a high-energy ration by a weekly increase of the proportions of the corn and grass silage mixture from 0 to 40 % of dry matter (DM) and concentrate feed from 0 to 60 % of DM within 6 weeks (wk). This diet was then maintained for further 9 wks. Body weight (BW, kg) and body condition score (BCS, according to the 5-scale by Edmonson et al. (1989) were monitored every 2 wks.

Blood samples from the jugular vein were collected monthly and scAT biopsies were taken from the tailhead region at the beginning of the experiment (0 wk), after 8 and 15 wks as described recently (Locher et al., 2014). Tissue samples were immediately snap frozen in liquid nitrogen to isolate DNA and RNA for quantitative PCR or were fixed in 4% paraformaldehyde (Roth, Karlsruhe, Germany) for histological evaluations.

Variables indicative for oxidative stress

Oxidative stress was determined in serum by the dROM tests (derivates of reactive oxygen metabolites) (dROM) using N,N-diethyl-para-phenylendiamine (DEPPD) as chromogenic substrate (Alberti et al.,2000) with the modifications given by Regenhard et al. (2014). The results are expressed as H_2O_2 equivalents.

Advanced oxidation protein products (AOPP) in plasma were determined by the modified spectrophotometric methods of Witko-Sarsat et al. (1998) and Celi et al. (2011). Different dilutions (6.25 to 100 μM) of Chloramin-T (Sigma-Aldrich) in PBS (pH 7.3) were used to generate standard curves, and PBS without Chloramin-T served as blank. Samples and standards were incubated with 40 μL pure acetic acid (Roth) for 5 min at room temperature (RT) and 20 μL potassium iodide (Sigma-Aldrich) was added to the standards. The absorption was measured spectrometrically at 340 nm (Genesys 10 UV) and AOPP concentrations are expressed in relation

to albumin concentrations (μmol/g), which were determined by an automatic analyzer system (Eurolyser CCA180, Eurolab) and were already reported by Dänicke et al. (2014).

The formation of lipid peroxides was measured in serum using a biochemical assay for thiobarbituric acid reactive substances (TBARS; BioAssay Systems) according to the manufacturer's protocol. In brief, 100 μL serum was mixed with 200 μL trichloroacetic acid (10%), incubated for 15 min on ice and centrifuged at 18,000 x g for 5 min at 4 °C. Different dilutions of malondialdehyde in H_2O (0.25 - 4.5 μM) served as standard curve. For the color reaction, 200 μL TBA reagent was added to the samples and standards and heated at 100 °C for 60 min. TBARS were determined photometrically (excitation: 560 nm; emission: 585 nm; FluoroMax, Spex).

Histological evaluations

Immunohistochemistry on paraffin embedded AT sections (12 μm) was performed according to protocols developed earlier (Häussler et al., 2013). Immunostaining of HIF-1α was based on a polyclonal rabbit antiserum against human HIF-1α (1:200; GTX 127309; Genetex). For detecting VEGF-R2, a polyclonal rabbit anti-VEGF-R2 antibody (1:100; bs-0565R; Bioss Inc.) was used. Specific primary antibodies were incubated overnight at 4 °C. Afterwards, the sections were incubated with horseradish peroxidase-labelled goat-anti-rabbit IgG (Southern Biotech; 1:200; 30 min at RT). Immunostaining was achieved with 3-amino-9-ethylcarbazol (Toronto Research Chemicals Inc.) and counterstaining was performed by Mayer's Haemalaun (Merck Millipore). Bovine placenta (VEGF-R2) and kidney (HIF-1α) served as negative and positive control. For negative controls the primary antibodies were replaced by PBS.

Apoptosis was determined by a modified terminal deoxynucleotide transferase-mediated dUTP nick-end-labeling (TUNEL) assay (Gavrieli et al., 1992) as described recently (Häussler et al., 2013). Bovine lymph node samples from slaughterhouse animals served as negative and positive controls.

The sections were evaluated at 200-fold magnification by light microscope (Leica DMR, Leica Microsystems) equipped with a JVC digital color camera KY-F75U (Hachioji Plant of Victor Company). For each section, 10 randomly selected fields (350 x 450 μm) were captured and the

number of positive stained cells as well as the total cell number was counted. Results are presented as the mean percentage of positive cells per total cell number in the evaluated fields. Adipocyte areas (µm²) were determined in 100 randomly selected adipocytes per histological sections as described recently (Akter et al., 2011).

Gene expression assays

Extraction of total RNA and cDNA synthesis was done as described by Saremi et al. (2012). Quantitative PCR analysis was carried out with a Mx3000P cycler (Stratagene). Each run included an inter-run calibrator, a negative template control for qPCR, a negative template control and a no reverse transcriptase control of cDNA. The quantification of samples was performed against a cDNA standard curve with serial dilutions. The results of the genes of interest (HIF-1α, VEGF-R2, VEGF-A, NRF1, NRF2, TFAM, PGC-1α) were normalized based on the geometric mean of the amplified reference genes Marvel domain containing 1 (MARVELD1), eucariotic translation initiation factor 3 (EIF3K) and lipoprotein receptor-related protein 10 (LRP10). Primer sequences and accession numbers are given in Table 1.

DNA isolation and Multiplex qPCR

The mtDNA copy number per cell was assessed by multiplex qPCR (Cawthon, 2009). Total genomic DNA from scAT biopsies was extracted by a commercially available DNA Isolation kit (PowerPlant Pro DNA Isolation Kit; MOBIO) according to the manufacture's protocol. Purity and concentration of total DNA were measured at 260 nm and 280 nm by Nanodrop 1000 (peQLab Biotechnology) and the integrity of DNA was assessed by gel electrophoresis. To determine the relative quantity of mtDNA products, total DNA was mixed with two sets of primers: one amplified 12S rRNA, a sequence specific in the mitochondrial genome, the second one was specific for bovine ß-globin, a housekeeping gene acting as a nuclear control with a known copy number of two per cell (Brown et al., 2012). Primer sequences of ß-globin were adopted from Brown et al. (2012). The specificity of both primers was controlled using gel electrophoresis. Multiplex qPCR was set by adding 10 µL Dynamo SYBR Green (ThermoScientific) and 0.12 µL ROX as passive reference dye (ThermoScientific), both forward and reverse primers (1 µL each; Table 1) and nuclease free water to the DNA samples to a final volume of 20 µL. The PCR conditions were modified according to the procedure reported by Brown et al. (2012). A DNA standard curve was used to estimate PCR efficiency and a pooled

DNA sample served as interrun calibrator. Relative mtDNA copy numbers were calculated
according to Nicklas et al. (2004):

Relative mtDNA copy number per cell =

ß-globin copy number x PCR-efficiency $^{-(Ct12S\ rRNA\ –\ Ct\ ß-globin)}$

Table 1 Sequences of the primer used for quantifying target and reference genes.

	Forward Primer Sequence (5'-3')	**Reverse Primer Sequence (5'-3')**	**Acc. no.**
Gene			
VEGF-A	CATGGATGTCTACCAGCGCA	TGTTGAACTCCTCAGTGGGC	NM_174216.1
VEGF-R2	CCAGACCATGCTTGACTGCT	TGCCATCCTGTTGAGCGTTA	NM_001110000.1
HIF-1α	TTGGCAGCAATGACACAGAGA	TCTTGGTTGAGTGCAGGGTC	NM_174339
PGC-1α	GAAGGCAATTGAAGAGCGCC	TCGACCTGCGCAAAGTGTAT	NM_177945.3
TFAM	ATGCTTACAGGGCAGACTGG	AGCTTTACCTGTGATGTGCCA	NM_001034016.2
NRF1	CCCAAACTGAGCACATGG	GTTAAGTATGTCTGAATCGTC	NM_001098002.2
NRF2	TTCCAGCATCAGTGCAGTCT	CTGGCCATTGTTTCCTGTTC	NM_001075437.2
MARVELD1	GGCCAGCTGTAAGATCATCACA	TCTGATCACAGACAGAGCACCAT	NM_001101262
EIF3K	CCAGGCCCACCAAGAAGAA	TTATACCTTCCAGGAGGTCCATGT	NM_001034489
LRP10	CCAGAGGATGAGGACGATGT	ATAGGGTTGCTGTCCCTGTG	BC149232
MtDNA copy number			
12S rRNA	CGCGGTCATACGATTAACCC	AACCCTATTTGGTATGGTGCTT	NM_U01920.1
ß-globin	CGGCGGCGGGCGGCGCGGGCT GGCGGGAAGGCCCATGGCAAGA GG	GCCGGCCCGCCGCGCCCGTCCCGC CGCTCACTCAGCGCAGCAAAGG	

VEGF-A: Vascular endothelial growth factor A; VEGF-R2: Vascular endothelial growth factor receptor-2; HIF-1α:Hypoxia inducible factor 1 alpha; PGC-1α:Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; TFAM: Mitochondrial transcriptional factor A; NRF1: Nuclear respiratory factor 1; NRF2: Nuclear respiratory factor 1; MARVEL1: Marvel domain containing 1; EIF3K: Eucariotic translation initiation factor 3; LRP10: Lipoprotein receptor-related protein 10; 12S rRNA: Mitochondrially encoded 12S ribosomal RNA

Statistical analyses

Statistical analyses were performed using SPSS version 22.0 (SPSS Inc.). Data for all variables were tested for normal distribution using the Kolmogorov-Smirnov test and for homogeneity of variances by the Levene's test. Not normally distributed variables as well as mRNA values were log-transformed for statistical analyses and back transformed to the original scale after calculation. Data were analyzed using linear mixed models with "sampling dates" as fixed effect and "cow" as random effect and Bonferroni Post Hoc Test. Values are expressed as mean ± SEM or as median, 1st and 3rd quartile. Correlations were assessed by Spearman analysis. Results with a *P*-value ≤ 0.05 were considered to be significantly different and $0.05 < P ≤ 0.1$ was set as a trend.

RESULTS

The cows gained BW and BCS in the course of the whole experiment from 540 ± 57 to 792 ± 82 and 2.31 ± 0.35 to 4.53 ± 0.39, respectively (*P* ≤0.05).

The concentrations of dROM, AOPP and TBARS in plasma were determined to describe oxidative stress. From wk 0 to wk 15, the plasma concentrations of dROM and TBARS increased 2.5- and 2.2-fold (*P* ≤0.007), respectively (Figs. 1A and C), whereas the AOPP concentrations tended to decrease after the first biopsy (*P* =0.106; Fig. 1B).
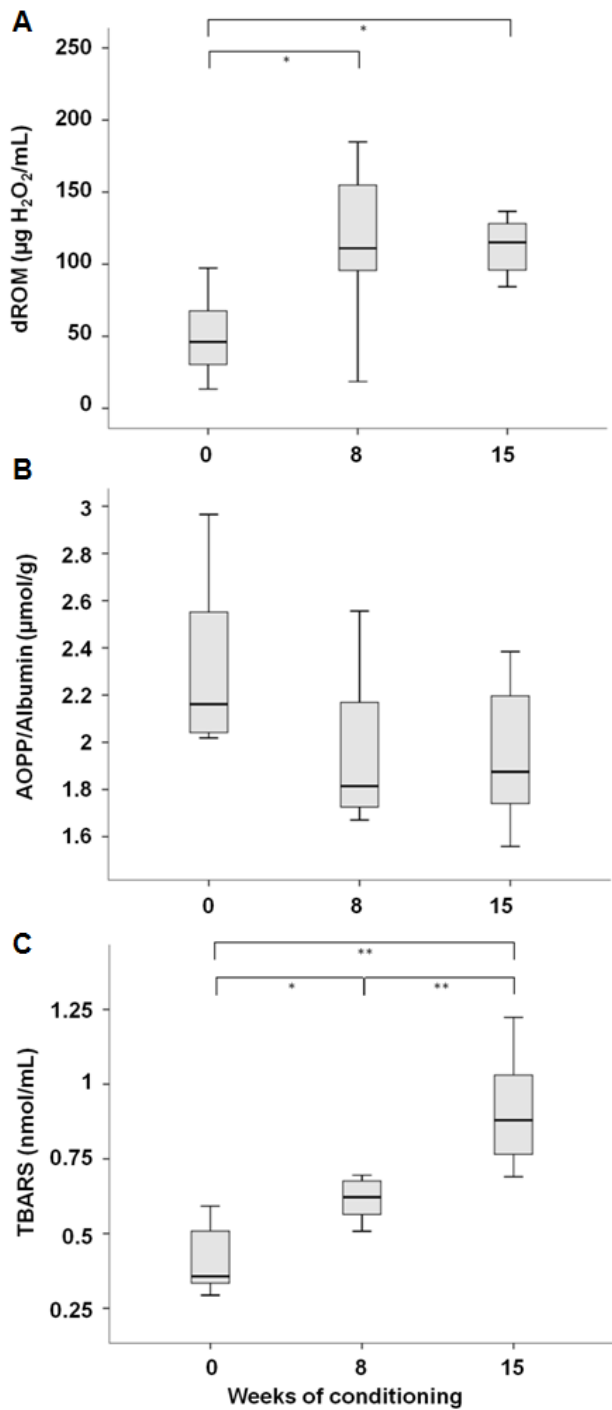
Figure 1 Time dependent changes of dROM (µg H_2O_2/mL; A), AOPP/Albumin (mol/g; B) and TBARS (nmol/mL; C) levels in serum from non-pregnant, non-lactating dairy cows at the beginning (wk 0), wk 8 and after 15 wk of the experiment. Cows were fed a diet with increasing portion of concentrate (reaching 60% of dry matter within 6 wk), that was then maintained for further 9 wk. Data are presented as medians, 1st and 3rd quartiles, and minimum/maximum values. Asterisks indicate significant differences between samplings after Bonferroni Post Hoc Test; *: $P < 0.05$; **: $P < 0.005$.

The abundance of mtDNA copies per cell was examined using multiplex qPCR. From wk 0 to wk 8, mtDNA copies per cell increased 4.7-fold (P <0.001) and remained constant from wk 8 to wk 15 (Fig. 2).
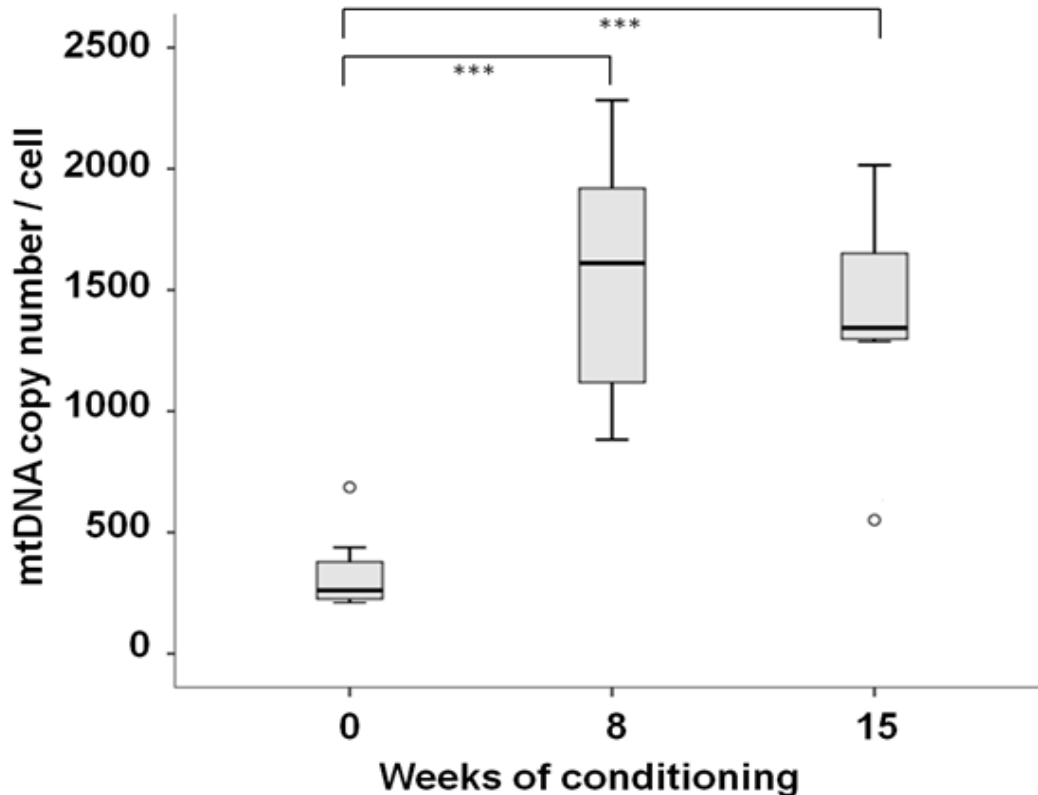
Figure 2 Mitochondrial DNA (mtDNA) copy number/cell in subcutaneous adipose tissue biopsies at the beginning (wk 0), wk 8 and wk 15 of the experiment. Non-lactating, non-pregnant dairy cows were fed a diet with increasing portion of concentrate (reaching 60% of dry matter within 6 wk). This diet was maintained for further 9 wk. Data are presented as medians, 1st and 3rd quartiles, and minimum/maximum values. Asterisks indicate significant differences between samplings after Bonferroni Post Hoc Test; ***: $P \leq 0.001$.

Immunohistochemical stainings of VEGF-R2 were found in the cytoplasm of cells in scAT as exemplarily demonstrated in Fig. 3A. The portion of VEGF-R2 positive cells increased throughout the whole conditioning period (1.2-fold, P =0.028). After a numerical decrease (1.1-fold) from the beginning of the experiment until wk 8, the expression of VEGF-R2 was elevated 1.4-fold (P =0.001) from wk 8 until the end of the experiment (Fig. 4A). The number of HIF-1α positive cells was determined by immunohistochemistry in scAT (Fig. 3B) and increased 3.3-fold

(P =0.003) from wk 0 to wk 15 (Fig. 4B). From experimental wk 0 to 8, HIF-1α protein levels increased 2.6-fold (P =0.045) and stagnated thereafter.
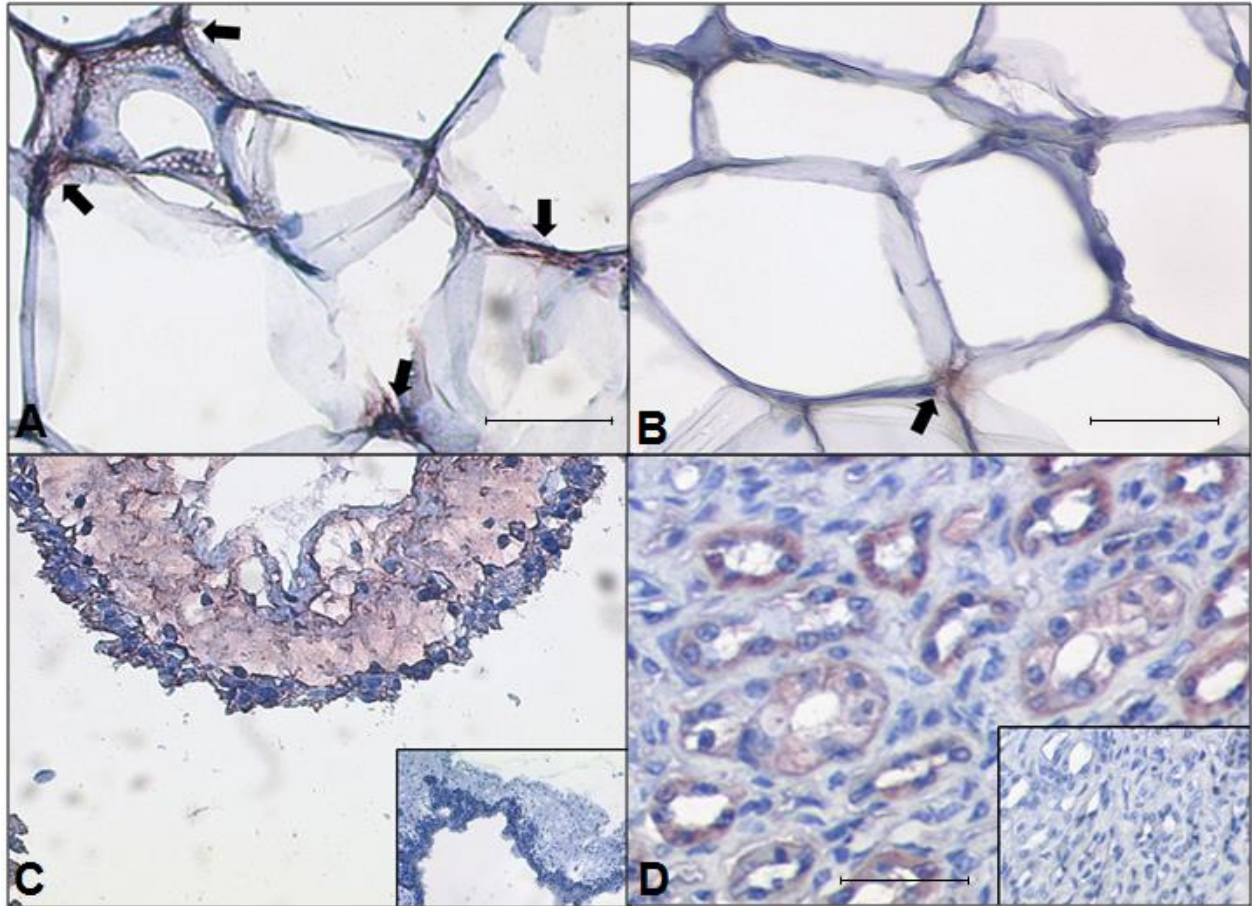
Figure 3 Examples of Vascular endothelial growth factor receptor 2 (VEGF-R2; A) and hypoxia inducible factor 1 alpha (HIF-1α; B) immunoreactivity in histological sections from subcutaneous adipose tissue. Positive cells appear as weak, red staining in the cytoplasm of scAT cells (marked with arrows). Bovine placenta was used as positive and negative control (C and D). Original magnification: 200-fold. Scale bars represent 100 μm.
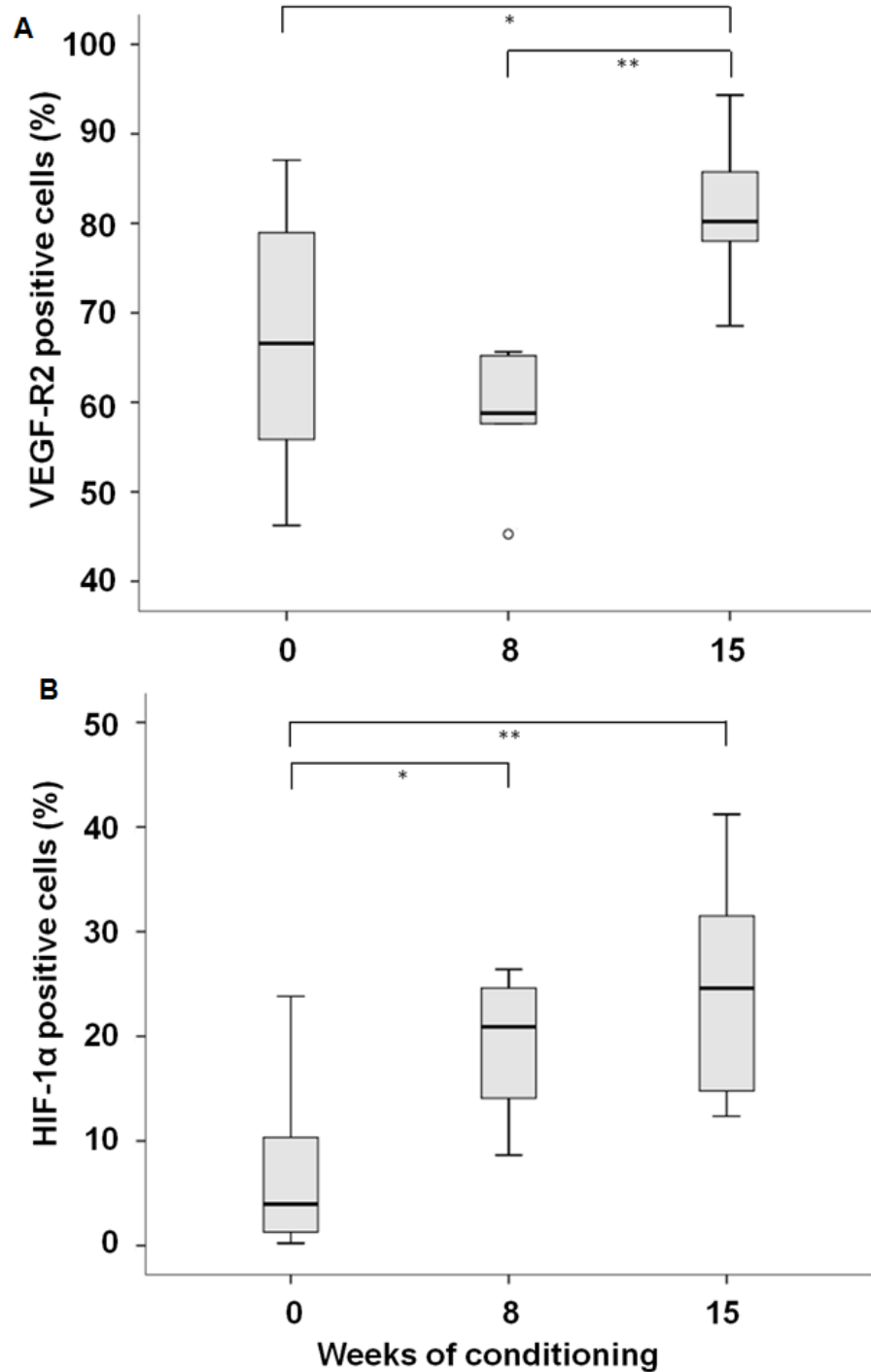
Figure 4 Portion of positive cells (%) for vascular endothelial growth factor receptor 2 (VEGF-R2; A) and hypoxia inducible factor 1α (HIF-1α; B) in subcutaneous adipose tissue at wk 0, 8 and 15 of conditioning. Non-lactating, non-pregnant dairy cows were fed a diet with increasing portion of concentrate (reaching 60% of dry matter within 6 wk). This diet was maintained for further 9 wk. Data are presented as median, 1st and 3rd quartiles, and minimum/maximum values. ○ = extreme value. Asterisks indicate significant differences between samplings after Bonferroni Post Hoc Test; *: $P < 0.05$; **: $P < 0.005$.

The apoptotic cell rate decreased 2.5-fold from wk 0 to 8 ($P = 0.026$) without any further changes until the end of the experiment (Fig. 5).
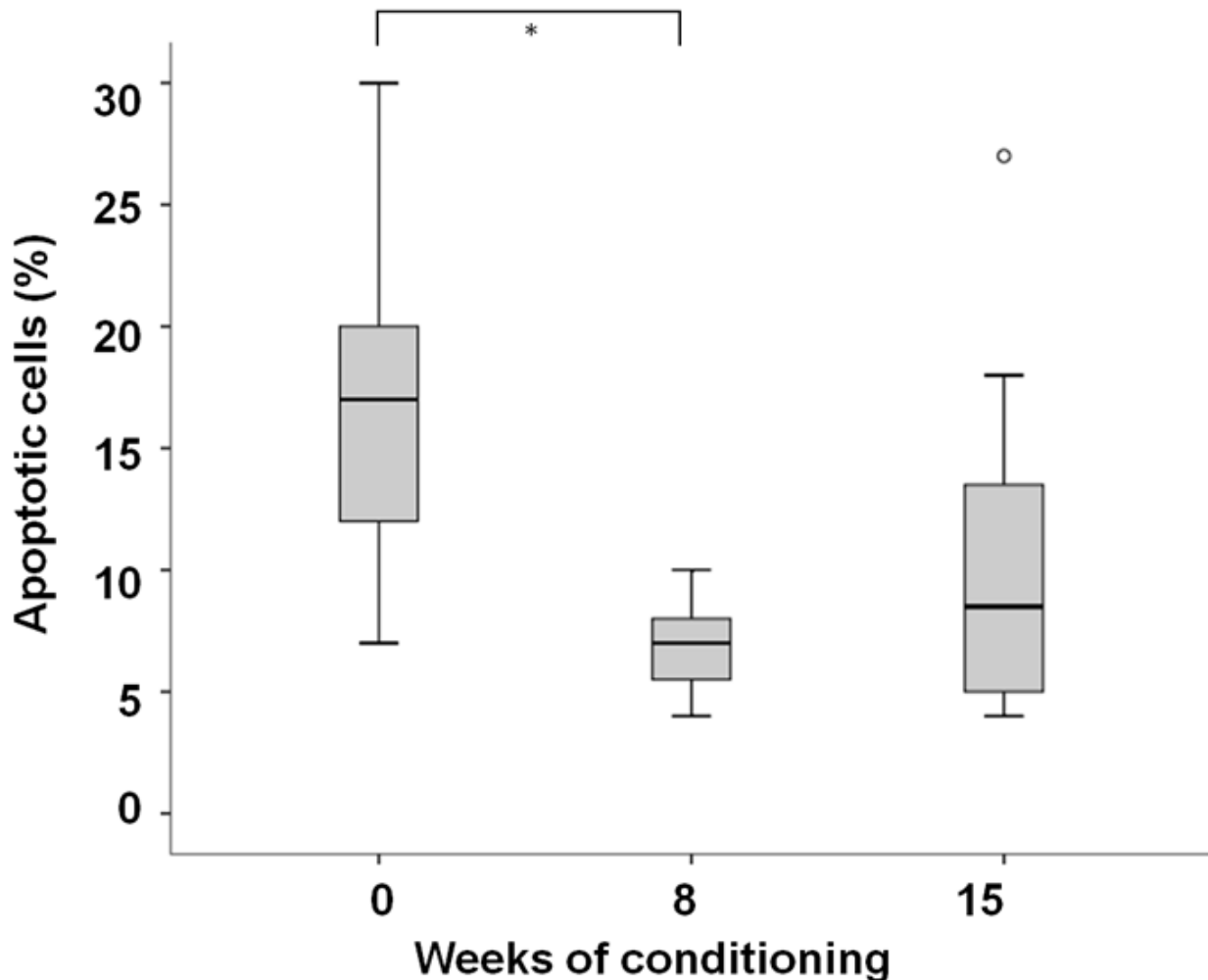
Figure 5 Portion of apoptotic cells (%) in subcutaneous adipose tissue from non-pregnant, non-lactating dairy cows at the beginning (wk 0), wk 8 and after 15 wk of the experiment. Cows were fed a diet with increasing portion of concentrate (reaching 60% of dry matter within 6 wk), that was then maintained for further 9 wk. Data are presented as median, 1st and 3rd quartiles, and minimum/maximum values. ○ = extreme value. Asterisk indicates significant differences between samplings after Bonferroni Post Hoc Test; *: P < 0.05.

The mRNA abundances of pro-angiogenic factors (VEGF-A, VEGF-R2 and HIF-1α) as well as genes related to mitochondrial biogenesis (NRF1, NRF2, PGC-1α and TFAM) in scAT are shown in Table 2. Expression of VEGF-A and VEGF-R2 tended to decrease from the beginning of the high-concentrate diet until the end of the experiment, whereas HIF-1α mRNA decreased 1.7-fold from wk 0 to 8 ($P = 0.037$). The mRNA abundances of NRF1, NRF2, TFAM remained

stable throughout the whole experiment, whereas PGC-1α abundances tended to increase 2.4-fold (P =0.087) from wk 0 to 15.

Table 2 Relative mRNA abundances (mean ± SEM) of angiogenic genes (VEGF-A, VEGF-R2, HIF-1α) and of mitochondrial biogenesis genes (NRF1, NRF2, PGC-1α, TFAM) in subcutaneous adipose tissue at 0, 8, and 15 weeks (wk) of conditioning. Non-lactating, non-pregnant cows were fed a diet with increasing amounts of concentrate until a portion of 60 % of concentrate (on a dry matter basis) was reached within 6 wk and continued on this diet for further 9 wk.

	Conditioning (wk)		
	0	8	15
Angiogenic genes			
VEGF-A	1.51 ± 0.31	1.11 ± 0.15	0.80 ± 0.09
VEGF-R2	1.30 ± 0.19	1.09 ± 0.12	0.78 ± 0.09
HIF-1α	1.38 ± 0.21^{a}	0.79 ± 0.09^{b}	$1.05 \pm 0.12^{a,b}$
Mitochondrial biogenesis genes			
NRF1	1.11 ± 0.18	0.81 ± 0.16	1.06 ± 0.11
NRF2	0.81 ± 0.16	0.91 ± 0.18	1.24 ± 0.12
PGC-1α	0.61 ± 0.19	0.95 ± 0.25	1.46 ± 0.4
TFAM	0.96 ± 0.2	1.01 ± 0.17	1.37 ± 0.14

Different letters between weeks of conditioning indicate significant differences ($P \le 0.05$).
VEGF-A: Vascular endothelial growth factor A; VEGF-R2: Vascular endothelial growth factor receptor-2; HIF-1α: Hypoxia inducible factor 1-alpha; PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; TFAM: Mitochondrial transcriptional factor A; NRF-1,-2: Nuclear respiratory factor 1,2

The coefficients of correlation between protein and mRNA expression of angiogenic variables (VEGF-A, VEGF-R2, HIF-1α) as well as mtDNA copy numbers and mitochondrial biogenesis genes (TFAM, PGC-1α) with indicators for oxidative stress (TBARS, dROM), body composition and blood variables are shown in Table 3. Neither NRF1, nor NRF2 and AOPP were associated with the aforementioned parameters.

Furthermore, mtDNA copy numbers were positively related to HIF-1α protein (ρ =0.658; P =0.001) and negatively to the number of apoptotic cells (ρ =-0.488; P =0.039). Moreover, HIF-1α mRNA was associated with VEGF-R2 mRNA (ρ =0.542; P =0.02).

Very strong correlations were observed between TBARS and BW (ρ =0.755; P <0.001) and BCS (ρ =0.877; P <0.001). Moreover, TBARS concentrations were moderately related to adipocyte areas (ρ =0.496; P =0.016) and insulin concentrations (ρ =0.587; P =0.003). In addition, dROM concentrations were positively related to BW (ρ =0.585; P =0.003), BCS (ρ =0.537; P =0.007) and adipocyte areas (ρ =0.488; P =0.018) and tended to be correlated with insulin concentrations (ρ =0.370; P =0.075).

Table 2 Relationships between angiogenic and mitochondrial biogenesis variables and indicators for oxidative stress, body condition as well as blood variables of non-pregnant, non-lactating dairy cows during the whole period of experimental over-conditioning.

	Angiogenesis					Mitochondrial biogenesis		
	Protein Expression		Gene Expression			Gene Expression		
	VEGF-R2	HIF-1α	VEGF-R2	HIF-1α	VEGF-A	mtDNA	TFAM	PGC1
Oxidative stress indices								
dROM	n.s.	$\rho = 0.380$	ρ = - 0.713	ρ = - 0.486	n.s.	ρ = 0.550	n.s.	n.s.
TBARS	ρ = 0.463	ρ = 0.446	ρ = - 0.550	n.s.	$\rho = - 0.430$	ρ = 0.447	$\rho = 0.406$	$\rho = 0.461$
Body condition variables								
BCS[**]	ρ = 0.516	ρ = 0.453	ρ = - 0.516	n.s.	ρ = - 0.588	ρ = 0.503	n.s.	$\rho = 0.409$
Body weight[**]	ρ = 0.512	ρ = 0.516	ρ = - 0.517	n.s.	ρ = - 0.672	ρ = 0.596	n.s.	$\rho = 0.443$
Adipocyte area[*]	n.s.	ρ = 0.455	n.s.	n.s.	ρ = - 0.478	$\rho = 0.388$	n.s.	$\rho = 0.439$
Blood variables								
Leptin[*]	$\rho = 0.362$	ρ = 0.548	ρ = - 0.498	n.s.	ρ = - 0.575	ρ = 0.707	$\rho = 0.434$	ρ = 0.492
Insulin[**]	n.s.	ρ = 0.482	n.s.	n.s.	n.s.	ρ = 0.616	n.s.	n.s.
NEFA[**]	n.s.	ρ = - 0.541	n.s.	n.s.	$\rho = 0.450$	$\rho = -0.381$	n.s.	n.s.

bold: $P \leq 0.05$; italicized: $0.05 < P \leq 0.1$; n.s.: not significant

[*]Data already published by Locher et al. 2014
[**] Data already published by Dänicke et al. 2014

DISCUSSION

The present study aimed to investigate the impact of an excessive fat accumulation in cows on key regulators of mitochondrial biogenesis, angiogenesis and oxidative stress. With the onset of lactation, over-conditioned cows mobilize more body reserves than lean cows and are therefore susceptible to develop health problems and metabolic disorders (Bernabucci et al., 2005; Roche et al., 2009). Obesity in humans is often related to dysfunctions in AT angiogenesis (Gealekman et al., 2011; Kabon et al., 2004) and mitochondrial biogenesis (Yin et al., 2014) as well as to the development of high levels of oxidative stress (Higdon and Frei, 2003). Therefore, we aimed to investigate whether these incidents occur in over-conditioned cows, independent from physiological changes related to parturition and lactation.

Increasing body condition in the present study was accompanied by elevated dROM and TBARS concentrations indicating enhanced oxidative stress (Bernabucci et al., 2005). However, unchanged plasma AOPP concentrations led to the assumption that excessive protein oxidation products were not generated. Oxidative stress was more pronounced in over-conditioned dry cows showing greater BCS loss at calving, compared to thin cows (Bernabucci et al., 2005). In the present study, insulin sensitivity tended to decrease by feeding a high energy diet as shown by Locher et al. (2014). Moreover, increasing insulin concentrations were associated with TBARS and tended to be related to the dROM concentrations.

Excessive accumulation of ROS in adipocytes can impair mitochondrial function (Kusminski and Scherer, 2012) and may further result in insulin insensitivity as detected in human adipocytes (Wang et al., 2013). Therefore, we aimed to test whether the mtDNA content and mitochondrial biogenesis were affected in response to an excessive energy intake and increased oxidative stress in scAT. Increasing mtDNA copies might be an adaptive response mechanism to compensate mtDNA damage caused by increased ROS (Lee et al., 2000). The positive relationship between mtDNA copy number and oxidative stress variables indicate that besides their importance for cellular energy metabolism, mitochondria are the major source of ROS production (Sawyer and Colucci, 2000). Vice versa, increasing ROS may cause more oxidative damage to mitochondria and other cell organelles (Al-Kafaji and Golbahar, 2013), which might impair cellular energy metabolism and finally result in cell senescence or apoptosis (Chen et al., 1998; Passos and von Zglinicki, 2005).

The mRNA abundances of key transcription factors of mitochondrial biogenesis, i.e. PGC-1α, NRF1, NRF2 and TFAM, which might control the amount and function of mtDNA in AT mitochondria (Villarroya et al., 2009) were determined and related to increased ROS production. Although PGC-1α is known to induce NRF1, NRF2 and TFAM (Puigserver et al., 1998), the mRNA abundances of these transcription factors remained unchanged, while PGC-1α tended to increase with increasing body condition. Gene expression of transcription factors for mitochondrial biogenesis might change after prolonged enhanced oxidative stress levels as suggested for rats suffering from chronic cholestasis (Arduini et al., 2011).

In the present study, mtDNA copy number was positively associated with BCS and BW and negatively related to decreasing NEFA concentrations. Furthermore, the positive association between mtDNA copies and circulating leptin, an adipokine related to BCS and adipocyte sizes in cattle (Delavaud et al., 2002; Ehrhardt et al., 2000), indicates a role of mtDNA content in lipogenesis of bovine AT as proposed for humans (Kaaman et al., 2007).

Large adipocytes require more mitochondria to meet the increased ATP demand of the larger cell (Yin et al., 2014). However, in obese humans (BMI >36.9) no further increase in mtDNA copy number with larger adipocytes was observed (Yin et al., 2014). In the present study, adipocyte sizes and the number of mtDNA copies were positively correlated, both increased 1.3-fold until wk 8 and stagnated thereafter (Locher et al., 2014). We suppose that stagnating mtDNA copy numbers would limit the energy supply in adipocytes.

Mitochondrial biogenesis was related to tissue oxygenation in the brain of neonatal rats (Lee et al., 2008). In general, hypoxia plays an important role in the context of obesity and obesity-related diseases; therefore, we hypothesized that AT from over-conditioned cows might suffer from hypoxia. Within AT, angiogenesis is adapted to hypertrophic adipocytes to ensure sufficient oxygen and nutrient supply (Lemoine et al., 2013). Enlarged adipocytes are prone to hypoxia and respond by activation of HIF-1α (Trayhurn et al., 2008). In the present study, increased HIF-1α positive cells were positively correlated with adipocyte sizes as well as with BW and BCS from the beginning of the experiment until wk 8. Due to the rapid enlargement of adipocyte sizes, the capillary density probably fails to meet the hypertrophy and results in insufficient nutrients and oxygen supply as it was found in mice (Pang et al., 2008) and humans (Karpe et al., 2002;

Pasarica et al., 2009). Due to hypoxia, adipocytes might undergo apoptosis or necrosis (Yin et al., 2009). However, despite increased HIF-1α the number of apoptotic cells decreased until wk 8. Increasing mtDNA copy number might act as a feedback mechanism to counterbalance the energy deficit in the cells (Carabelli et al., 2011).

Given that the number of HIF-1α positive cells stagnated from wk 8 until the end of the experiment, the increase of VEGF-R2 positive cells from wk 8 to 15 might respond to the hypoxic condition within scAT. In order to initiate remodeling of blood vessels HIF-1α enhances the expression of angiogenic growth factors, such as VEGF and its receptors in human skeletal muscle (Gorlach et al., 2001).

The positive association between the number of mtDNA copies per cell and HIF-1α protein expression in the present study might point to a compensation of the hypoxic condition through increased mtDNA as previously postulated for rats suffering from hypoxia in liver (Carabelli et al., 2011) and in brain (Lee et al., 2008). Albeit HIF-1α has been considered to be an important regulator of mitochondrial biogenesis in skeletal muscle (Mason et al., 2007), no association was observed between mRNA abundances of mitochondrial genes and HIF-1α mRNA in AT in the present study.

CONCLUSIONS

In summary, due to rapid fat accumulation, over-conditioned, non-pregnant and non-lactating cows were characterized by increased blood concentrations of markers for oxidative stress. Increasing numbers of mtDNA copies might improve the energy supply within expanding AT as a compensatory mechanism to oxidative stress. Vice versa increasing mitochondria generate more ROS leading to more mtDNA damage. However, no changes in the mRNA expression of transcription factors for mitochondrial biogenesis were observed. Local hypoxia accompanied by adipocyte growth may be counterbalanced by angiogenic remodeling of blood vessels.

ACKNOWLEDGEMENTS

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4   Manuscript II (submitted)

Mitochondrial DNA copy number and biogenesis in different tissues of early and late lactating dairy cows

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INTERPRETIVE SUMMARY

Mitochondrial DNA copy number and biogenesis in different tissues of early and late lactating dairy cows. *By Laubenthal et al.* In high-yielding dairy cows, key organs such as liver, mammary gland, and adipose tissue are continuously adapted to varying energy requirements during lactation. Therefore, we aimed to investigate the abundance and regulation of mitochondria, the main sites for cellular energy production, in blood and physiologically relevant tissues, during early lactation - the most challenging time for dairy cows - and late lactation. The mitochondrial DNA copy number, reflecting the abundance of mitochondria, was influenced by stage of lactation and varied between different tissues and blood in dairy cows.

ABSTRACT

Energy balance in dairy cows is variable in the course of lactation due to changes in voluntary feed intake and energy required for milk synthesis. In order to adapt to the demands of lactation, energy metabolism needs to be regulated and coordinated in key organs such as adipose tissue (AT), liver, and mammary gland. Mitochondria are the main sites of energy production in mammalian cells and their number varies depending on age, organ and physiological condition. The copy number of mitochondrias' own genome, the mitochondrial DNA (mtDNA), reflects the abundance of mitochondria within a cell and is regulated by transcriptional and translational factors. Environmental, physiological and energetic conditions alter during lactation and we thus hypothesized that these changes may influence the mtDNA copy number and the abundance of genes regulating mitochondrial biogenesis. Therefore, we aimed to provide an overview of mitochondrial biogenesis in liver, subcutaneous (sc) AT, mammary gland, and peripheral blood cells during early and late lactation in dairy cows. German Holstein cows (n=21) were fed according to their requirements and biopsies from scAT, liver, mammary gland as well as blood were collected in early and late lactation and assayed for relative mtDNA copy number determination and mRNA abundance of genes regulating mitochondrial biogenesis, such as the nuclear-respiratory factor 1 and 2 (NRF-1, NRF-2), mitochondrial transcription factor A (TFAM), and the peroxisome proliferator-activated receptor-gamma coactivator 1-alpha) PGC-1α).

The number of mtDNA copies increased from early to late lactation in all tissues, whereas the mtDNA copy number in peripheral blood was higher in early compared to late lactation. Comparing the number of mtDNA copies between tissues and blood in dairy cows, the highest mtDNA content was observed in liver. The mRNA abundances of genes related to mitochondrial biogenesis changed in a tissue-specific manner when comparing early versus late lactation. The mtDNA copy number was associated with transcriptional factors only in AT, suggesting a non-transcriptional regulation of mtDNA in the other tissues. We observed strong correlations between peripheral blood mtDNA and tissue mtDNA content in early lactation. Peripheral blood forms an appropriate medium to display the cellular content of mtDNA copy numbers and consequently the cellular energy status of tissues during early lactation.

INTRODUCTION

Increasing milk synthesis right after calving can dramatically change the energy metabolism of key organs, such as the mammary gland, liver, and adipose tissue (**AT**) of high-yielding dairy cows (Barber et al., 1997; Block et al., 2001). In many cases, an approximately 4-fold increase in energy demands in early-lactating cows compared to the pregnant and non-lactating state (Block et al., 2001), accompanied by a decrease in voluntary feed intake, results in a period of negative energy balance (**EB**). This energy deficit is primarily compensated by the mobilization of body reserves. Fatty acids are released in form of NEFA from AT into the circulation (Castañeda-Gutiérrez et al., 2009). In the mammary gland, NEFA are re-esterified into triacylglycerols (**TAG**) and secreted into the milk, whereas in the liver they are oxidized to CO_2, converted to ketone bodies or re-esterified into TAG and secreted in very low density lipoproteins. Excessive accumulation of liver TAG has been associated with diverse economically important diseases, including hepatic lipidosis and ketosis (Drackley, 1999). When lactation advances, the AT depots are re-filled by lipogenic processes in times of a positive EB in late lactation and the beginning of the dry period.

On a cellular level, alterations of nutrient and energy requirements of an individual coincide with changes of mitochondria, the main sites of high yielding ATP-generating reactions in mammalian cells. Their number varies depending on age, sex, organ, and physiological or pathological conditions (Fawcett, 1981). The mitochondrial DNA (**mt**DNA), i.e. the mitochondrias' own genome, reflects the abundance of mitochondria within a cell and can vary according to the energy demands of the cell (Al-Kafaji and Golbahar, 2013). Proliferation and differentiation of mitochondria and mtDNA are regulated and maintained by genes involved in the mitochondrial biogenesis, such as the nuclear-respiratory factor 1 and 2 (**NRF-1, NRF-2**), mitochondrial transcription factor A (**TFAM**) and the peroxisome proliferator-activated receptor-gamma coactivator 1-alpha (**PGC-1α**; Izquierdo et al., 1995). These transcription factors can control the amount and function of mtDNA in various tissues, e.g. human AT (Villarroya et al., 2009) as well as liver and muscle tissue (Pejznochova et al., 2010). The mtDNA decreases with age as shown for mice and humans, whereby mtDNA varies between different tissues (Barazzoni et al., 2000).

However, little is known about mitochondria and the impact of mitochondrial biogenesis in the mammary gland, liver, and AT as well as in peripheral blood cells of lactating dairy cows. Thus,

we aimed to analyze mtDNA copy numbers and genes regulating mitochondrial biogenesis in the aforementioned physiologically relevant key organs during early and late lactation. Parity is considered as an important factor influencing metabolic and hormonal changes (Theilgaard et al., 2002; Wathes et al., 2007); therefore, we hypothesized that primiparous (**PP**) and multiparous (**MP**) cows differ with respect to their mtDNA copy number due to different energy metabolism. In addition, mitochondrial activity enzymes, i.e. citrate synthase and cytochrome *c* oxidase, were measured in AT. Furthermore, we aimed to determine whether the mtDNA copy number in circulating blood may serve as an appropriate marker for tissue mtDNA content.

MATERIAL AND METHODS

Animals, Experimental Design, and Sample Collection

The animal trial was approved by the State Agency for Nature, Environment and Consumer Protection of North Rhine-Westphalia, Recklinghausen, Germany (File Number 84-02.05.20.12.160) and was conducted at the research station Frankenforst of the Faculty of Agriculture, University of Bonn, Königswinter, Germany. In total, 21 German Holstein cows (lactation number: 1 to 5) were fed diets according to the recommendations of the Society of Nutrition Physiology in Germany (GfE, 2001) with a partial mixed ration ($6.3 - 6.8$ MJ NE_L/kg DM) offered for *ad libitum* intake and concentrate feed (7.7 MJ NE_L/kg DM) depending on the individual's milk yield. Animals were housed in a freestall barn with adjacent milking parlor and were milked twice per day. Based on the equations published by GfE (2001), the NE_M, NE_L, and milk energy concentration were calculated as follows:

NE_M (MJ of NE_L/d) = 0.293 x kg $BW^{0.75}$

Milk energy concentration (MJ of NE_L/kg) = 0.38 x fat (%) + 0.21 x protein (%) + 0.95

NE_L = Milk energy concentration (MJ of NE_L/kg) x Milk yield (kg/d)

Fat and protein content of the milk were given by fixed values of 4% fat and 3.4% protein (GfE, 1991).

The net energy balance was calculated with the following equation:

Net energy balance (MJ of NE_L/d) = energy intake (MJ of NE_L/d) – [NE_M (MJ of NE_L/d) + NE_L (MJ of NE_L/d)]

Body weight (**BW**, kg), milk yield (kg) and feed intake (kg) were recorded daily. For EB and milk yield, the weekly means were used. Body condition scores (**BCS**, according to the 5-scale system (Edmonson et al., 1989)) were monitored on the day of the biopsies.

Blood samples were collected from the jugular vein at the day of the biopsy immediately before sedating cows and were kept on ice. To obtain serum and heparin-plasma, samples were centrifuged (15 min, 3,000 x g, 4°C) and stored at -80°C until analyzes. Blood DNA was isolated from whole heparin-blood.

Biopsies from subcutaneous (**sc**) AT of the tailhead region, liver, and mammary gland were taken in early (between 21 and 28 DIM) and late lactation (between 245 and 252 DIM). Prior to the biopsies animals were sedated by intravenous injection of 1 mL Xylazine (2%, Serumwerk Bernburg AG, Germany).

The biopsy sites were washed, shaved and sterilized with 70% ethanol. The biopsy area was locally anaesthetized by sc injection of lidocaine (liver: 10 mL; mammary gland: 1 mL; scAT: 20 mL; 2%; Bela-Pharm GmbH & Co. KG, Vechta, Germany).

Liver biopsies were obtained by liver puncture in the 10th intercostals space and 60 - 80 mg tissue was taken using a 12 g x 20 cm Core Tissue Biopsy Needle with a Bard®Magnum®Biopsy instrument (BARD MAGNUM, Covington, GA). For mammary gland biopsies, a 0.2 - 0.3 cm skin incision was made and two mammary tissue cores (30 - 60 mg each) were taken using a Core Tissue Biopsy Needle (12 g x 10 cm; BARD). The 2nd biopsy was made on the contralateral udder side of the 1st biopsy. For the scAT biopsies, a 5.0 cm skin incision was made in the region of the tailhead and scAT from the underlying fat layer was collected. The 2nd biopsy was made on the contralateral side of the 1st biopsy.

All biopsy samples were rinsed in sterile saline, immediately snap frozen in liquid nitrogen and stored at -80°C until further analyses. The areas of the incisions were treated with a oxytetracyclin-hydrochloride spray (Intervet Deutschland GmbH, Unterschleißheim, Germany).

Gene expression assays

Extraction of total RNA and synthesis of cDNA was done according to Saremi et al. (2012). Quantitative PCR (qPCR) analysis was carried out using a Mx3000P cycler (Stratagene, Agilent Technologies, CA, USA). Each run included an inter-run calibrator, a negative template control for qPCR, and a negative template control as well as a no reverse transcriptase control for cDNA. The quantification of samples was performed against a cDNA standard curve with serial

dilutions. The results of the genes of interest (*NRF-1*, *NRF-2*, *TFAM*, *PGC-1α*) were normalized based on the geometric mean of the amplified reference genes. Selection of the most stable reference genes for the different tissues was done as described by Hosseini et al. (2010) using qBASE[plus] 2.0 (Biogazelle, Ghent, Belgium); *EIF3K* (eucariotic translation initiation factor 3) and *EMD* (emerin) were quantified for liver samples, *POLR2A* (RNA polymerase II) and *EMD* were selected for AT samples and *LRP10* (lipoprotein receptor-related protein 10) and *HPCAL* (hippocalcin-like 1) were used for mammary gland samples.

DNA isolation and Multiplex qPCR

The number of mtDNA copies per cell was assessed by multiplex qPCR (Cawthon, 2009). Total genomic DNA from scAT biopsies was extracted by a commercially available DNA Isolation kit (PowerPlant® Pro DNA Isolation Kit; MOBIO**,** Carlsbad, CA) according to the manufacture's protocol. Genomic DNA from whole blood, liver and mammary gland was isolated using the Wizard Genomic DNA Purification Kit (Promega, Mannheim, Germany) according to the manufacturer's protocol. The concentration and purity of total DNA were controlled by absorbance readings at 260 nm and 280 nm using the Nanodrop 1000 (peQLab Biotechnology, Erlangen, Germany) after gel electrophoresis to assess the integrity of DNA. 10 ng/μL of total DNA were mixed with two pairs of primers. To determine the relative quantity of mtDNA products, the primer pair amplifying the 12S rRNA gene, a sequence specific in the mitochondrial genome and the primer pair being specific to the bovine ß-globin gene, a housekeeping gene that acts as a nuclear control gene with a known copy number of two per each cell were used (Brown et al., 2012). The specificity of both primers was controlled by gel electrophoresis. A multiplex qPCR was set up by adding 10 μL Dynamo SYBR Green (Thermo Scientific, Rockford, IL) and 0.12 μL ROX as passive reference dye (Thermo Scientific), both (12S rRNA and ß-globin) forward and reverse primers (1 μL each; Table 1) and nuclease free water to the DNA samples to a final volume of 20 μL. The PCR conditions were modified to the protocol reported by Brown et al. (2012). A DNA standard curve was used to estimate PCR efficiency for each qPCR run and a pooled DNA sample served as inter-run calibrator. The relative mtDNA copy numbers versus nuclear DNA were calculated according to Nicklas et al. (2004):

Relative mtDNA copy number/cell = ß-globin copy number[*] x PCR-efficiency $^{-(Ct\ 12S\ rRNA\ -\ Ct\ ß\text{-}globin)}$

[*]ß-globin copy number = 2 copies per cell

Table 1 Sequences of the primers used for quantification of target and reference genes.

	Forward Primer Sequence (5'-3')	Reverse Primer Sequence (5'-3')	Acc. no.
Gene			
PGC-1α	GAAGGCAATTGAAGAGCGCC	TCGACCTGCGCAAAGTGTAT	NM_177945.3
TFAM	ATGCTTACAGGGCAGACTGG	AGCTTTACCTGTGATGTGCCA	NM_001034016.2
NRF-1	CCCAAACTGAGCACATGG	GTTAAGTATGTCTGAATCGTC	NM_001098002.2
NRF-2	TTCCAGCATCAGTGCAGTCT	CTGGCCATTGTTTCCTGTTC	NM_001075437.2
EMD	GCCCTCAGCTTCACTCTCAGA	GAGGCGTTCCCGATCCTT	NM_203361
EIF3K	CCAGGCCCACCAAGAAGAA	TTATACCTTCCAGGAGGTCCATGT	NM_001034489
HPCAL	CCATCGACTTCAGGGAGTTC	CGTCGAGGTCATACATGCTG	NM001098964
LRP10	CCAGAGGATGAGGACGATGT	ATAGGGTTGCTGTCCCTGTG	BC149232
POLR2A	GAAGGGGGAGAGACAAACTG	GGGAGGAAGAAGAAAAAGGG	X63564
MtDNA copy number			
12S rRNA	CGCGGTCATACGATTAACCC	AACCCTATTTGGTATGGTGCTT	NM_U01920.1
ß-globin[1]	GGCGGCGGGCGGCGCGGGCTGGGC	GCCGGCCCGCCGCGCCCGTCCCGC	
	GGAAGGCCCATGGCAAGAAGG	CGCTCACTCAGCGCAGCAAAGG	

PGC-1α: Peroxisome proliferator-activated receptor gamma coactivator 1-alpha; TFAM: Mitochondrial transcriptional factor A; NRF-1: Nuclear respiratory factor 1; NRF-2: Nuclear respiratory factor 1; EMD: Emerin; EIF3K: Eucariotic translation initiation factor 3; HPCAL: Hippocalcin-like 1; LRP10: Lipoprotein receptor-related protein 10; POLR2A: RNA polymerase II; 12S rRNA: Mitochondrially encoded 12S ribosomal RNA
[1] Primer sequences adopted from Brown et al., (2012).

Citrate synthase and cytochrome c oxidase activity

For preparation of AT extracts, scAT biopsies were homogenized in two volumes of HEPES buffer [10 mM; pH 7.4 with complete protease inhibitor cocktail (one tablet/10 mL buffer; Roche, Mannheim Germany)] using a homogenizer (Precellys 24, Peqlab, Erlangen, Germany). Homogenates were centrifuged (twice at 14,000 x *g*, 10 min, 4°C) and the fat layer was removed. In AT extracts, the activities of citrate synthase (**CS**; Kit CS0720; Sigma-Aldrich, St. Louis, MO) and cytochrome *c* oxidase (**Cox**; Kit CYTOCOX1; Sigma-Aldrich) were determined. Total protein in AT extracts was quantified by Bradford assay (Roti®-Nanoquant K880, Roth, Karlsruhe). For each sample 1.37 µg protein was used for both measurements. CS and Cox activity was determined in triplicates. The reaction of CS was based on the formation of 2-nitro-5-thiobenzoic acid and the absorbance was measured every 15 s for 3 min spectrophotometrically at 412 nm (28°C) using a microplate reader (Synergy H1, BioTek, Winooski, VT) as described in the manufactures instructions. The oxidation of ferrocytochrome *c* by Cox was measured at 550 nm every 15 s for 3 min using a microplate reader (Synergy H1) following the manual.

For positive controls, AT extracts were replaced either by CS or Cox control enzyme, whereas for negative control the sample was replaced by H_2O. Data are presented as nmol/min/mg of protein.

Analysis of blood variables

BHBA, NEFA, albumin, total protein, glucose, cholesterol, triglycerides, aspartate aminotransferase (ASTA), gamma-glutamyltransferase (GGT) and glutamate dehydrogenase (GLDH) were determined in serum samples by an automatic clinical chemistry analyzer (Eurolyser CCA180, Eurolab, Hallein, Austria).

Variables indicative for oxidative stress

Oxidative stress was characterized in serum by assessing derivates of reactive oxygen metabolites (**dROM**) by a spectophotometric tests using N,N-diethyl-para-phenylendiamine as chromogenic substrate (Alberti et al., 2000) according to the modified protocol of Regenhard et al. (2014). The resulting values were calculated from the standard curves by linear regression and were expressed as H_2O_2 equivalents.

The formation of lipid peroxides was measured in serum using a biochemical assay kit for thiobarbituric acid reactive substances (**TBARS**; BioAssay Systems, Hayward, CA) according to the manufacturer's protocol. In brief, 100 µl sera were mixed with 200 µl 10% trichloroacetic acid, incubated for 5 min on ice and centrifuged at 18,000 x *g* for 6 min at 4°C. For the standard curve, different dilutions (2 µM to 0.125 µM) of malondialdehyde in H_2O were used. For the color reaction, 200 µl thiobarbituric acid were added to the samples and standards and heated at 100 °C for 60 min. Optical densities were measured at 515/535 nm by photometer (Synergy H1; BioTek, Winooski, VT). The TBARS concentrations (µmol/L) were calculated according the manufactures protocol.

Measurement of hepatocyte area

Liver biopsies were snap frozen in tissue freezing medium (Leica, Wetzlar, Germany) and stored at -80°C until cutting. Liver (6 µm) sections were cut using a cryostat (Leica) and mounted on SuperFrost® Plus slides (Menzel, Braunschweig, Germany). Frozen tissue sections were fixed in acetone, stained with hematoxylin (Merck, Darmstadt, Germany) after drying, and mounted with Kaiser`s glycerol gelatin (Merck). Hepatocyte area (µm²) was measured from each 50 hepatocytes per section. From each stained section, 5 random images were taken at 100×

magnification on a light microscope (Leica DMR, Leica Microsystems, Wetzlar, Germany) with a JVC digital color camera KY-F75U (Hachioji Plant of Victor Company, Tokyo, Japan) The hepatocyte area was measured by DISKUS software (4th version; Hilgers, Königswinter, Germany) and averaged afterwards.

Statistical analyses

Statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL). Data for all variables were tested for normal distribution using the Kolmogorov-Smirnov test and for homogeneity of variances by the Levene's test. Not normally distributed variables as well as mRNA values were log transformed and back transformed to the original scale after calculation. With regard to the homogeneity of variances, differences between early and late lactation were compared by the paired Students' T-test ($P \leq 0.05$) and differences between tissues were analyzed using the non-paired Students' T-test ($P \leq 0.05$). A mixed model was used to determine specific variations between animals of different parities with "lactation number" as fixed and "cow" as random effect and to analyze energy balance and milk yield with Bonferroni Post Hoc Tests. All values are expressed as means ± SEM. Correlations were assessed by Spearman analysis. Results with a *P*-value ≤ 0.05 were considered significant and $0.05 < P \leq 0.1$ was set as a trend.

RESULTS

Body condition and blood variables

The results of variables describing body condition as well as blood variables are shown in Table 2. From early to late lactation, BW and BCS increased 1.1-fold (*P* =0.002) and 1.3-fold (*P* <0.001), respectively. Net EB and milk yield increased during the course of lactation (*P* <0.001, Fig. 1), whereas energy requirements decreased at 22% from early to late lactation (*P* =0.004; Table 2). In addition, NEFA and BHBA concentrations decreased at 51% (*P* <0.001) and at 38% (*P* =0.002), respectively, from early to late lactation (Table 2). The serum concentrations of dROM and TBARS did not differ between early and late lactation. Neither the area of hepatocytes nor of adipocytes was affected by the time point of lactation. Serum concentrations of cholesterol, triglycerides, GGT, and GLDH were significantly increased from early to late lactation, whereas no time effect was observed for albumin, total protein, glucose, and ASAT.

Fig. 1
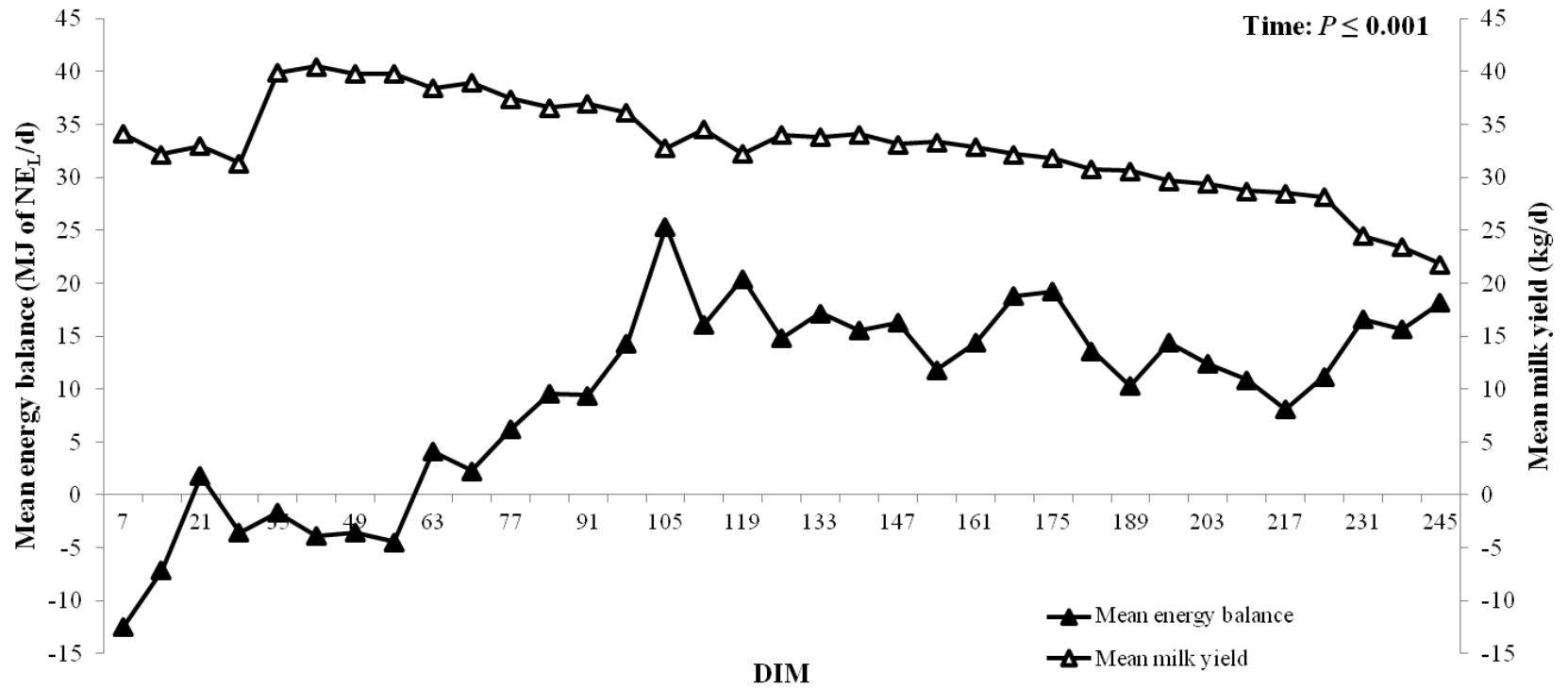
Figure 1 Development of net energy balance (means) and milk yield (means) in primiparous and multiparous dairy cows (n = 21) from 7 DIM to 245 DIM. P ≤0.05.

Table 2 Body condition, performance, hepatocyte area, as well as blood variables of dairy cows (n = 21) during early (21 to 28 DIM) and late (245 to 252 DIM) lactation. Means ± SEM.

	Lactation			
	Early		Late	
Energy balance (MJ NE_L/d)	-6.89 ± 3.83[a]		17.4 ± 4.31[b]	
Milk yield (kg/d)	33.2 ± 1.72[a]		22.8 ± 1.33[b]	
Body weight (kg)	627 ± 12.8[a]		657 ± 14.1[b]	
BCS	2.4 ± 0.1[a]		3.0 ± 0.1[b]	
Energy requirements (MJ NE_L/d)	132 ± 8.3[a]		103 ± 5.7[b]	
Hepatocyte area (µm²)	213 ± 6.15		224 ± 5.88	
Blood variables				
NEFA (mmol/L)	0.49 ± 0.04[a]		0.24 ± 0.02[b]	
BHBA (mmol/L)	0.92 ± 0.09[a]		0.57 ± 0.06[b]	
dROM (µg H_2O_2 equivalents/mL)	76.9 ± 7.40		82.0 ± 8.3	
TBARS (µmol/L)	0.22 ± 0.02		0.22 ± 0.17	
Albumin (g/L)	37.0 ± 0.93		37.7 ± 0.60	
Total protein (g/L)	71.9 ± 1.26		73.8 ± 1.16	
Glucose (mg/dL)	52.2 ± 2.98		56.9 ± 2.75	
Cholesterol (mg/dL)	115 ± 5.42[a]		183 ± 6.95[b]	
Triglycerides (mg/dL)	8.26 ± 0.61[a]		10.1 ± 0.68[b]	
ASAT (U/L)	81.3 ± 3.80		90.2 ± 6.84	
GGT (U/L)	20.9 ± 1.79[a]		30.0 ± 3.92[b]	
GLDH (U/L)	15.4 ± 2.32[a]		19.3 ± 1.93[b]	

Different letters indicate significant differences between early and late lactation. $P \leq 0.05$.
dROM: Derivates of oxygen metabolites; TBARS: Thiobarbituric acid reactive substances; ASTA: aspartate aminotransferase; GGT: gamma-glutamyltransferase; GLDH: glutamate dehydrogenase

Tissue-specific and lactation driven differences in mtDNA copy number

The number of mtDNA copies per cell was determined in scAT, liver, and mammary gland as well as in peripheral blood in early and in late lactation. In late lactation, blood mtDNA copy numbers were almost half of the amount present in early lactation (P =0.008; Fig. 2A). Within scAT, mtDNA copy number increased 1.7-fold (P <0.001) from early to late lactation (Fig. 2B). Furthermore, the mtDNA copies in liver rose 1.2-fold (P <0.001; Fig. 2C) and in mammary gland

the mtDNA copy number tended to increase 1.3-fold ($P = 0.057$; Fig. 2D) from early to late lactation.

When comparing the number of mtDNA copies between the investigated tissues and peripheral blood, the greatest mtDNA content was observed in liver being about 6.5-fold, 5.2-fold, and 5.8-fold higher ($P < 0.001$) than in AT, mammary gland, and peripheral blood, respectively.
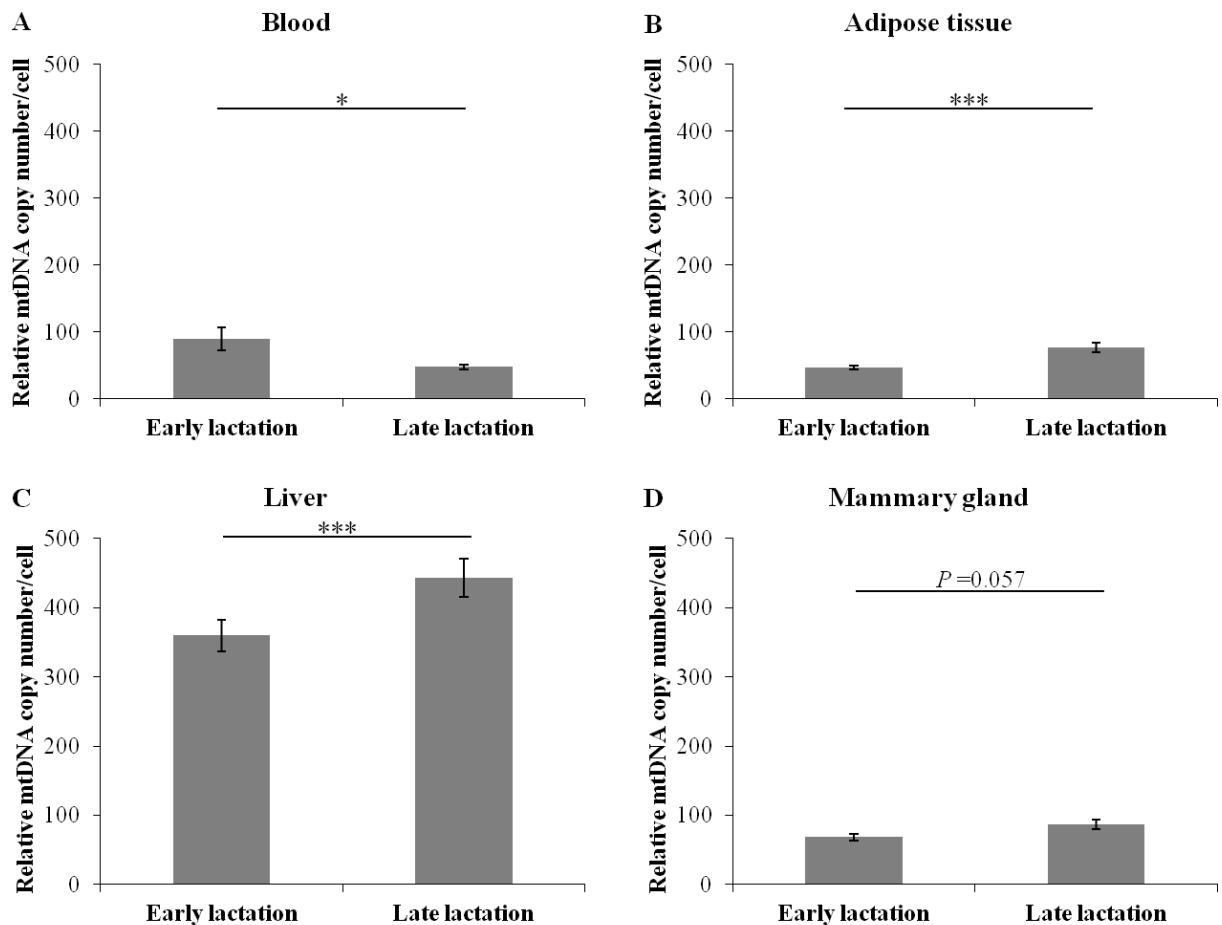
Figure 2 Mitochondrial DNA (mtDNA) copy numbers per cell (means ± SEM) in different tissues in early and in late lactation. Blood (A; $n = 21$), adipose tissue (B; $n = 21$), liver (C; $n = 21$) and mammary gland (D; $n = 19$) biopsies were taken in early (21 to 28 DIM) and late (245 to 252 DIM) lactation. Asterisks indicate significant differences between early and late lactation.*: P < 0.05; ***: P ≤ 0.001.

Citrate synthase activity and cytochrome c oxidase activity in adipose tissue

The activity of CS and Cox in AT are presented in Figure 3. In late lactation, scAT CS activity was around 2.4-fold higher compared to early lactation (P <0.001). Over all data, CS activity was positively related to AT mtDNA copy numbers (ρ = 0.689; P <0.001). This positive correlation was limited to late lactation (ρ = 0.622; P = 0.003).

From early to late lactation, the activity of Cox was increased 1.9-fold (P =0.002). Irrespective of lactation, Cox activity was positively associated with mtDNA copy numbers in AT (ρ = 0.491; P =0.001), whereas no relation was observed when considering early and late lactation separately. The ratio between Cox and CS was the same in early and late lactation.
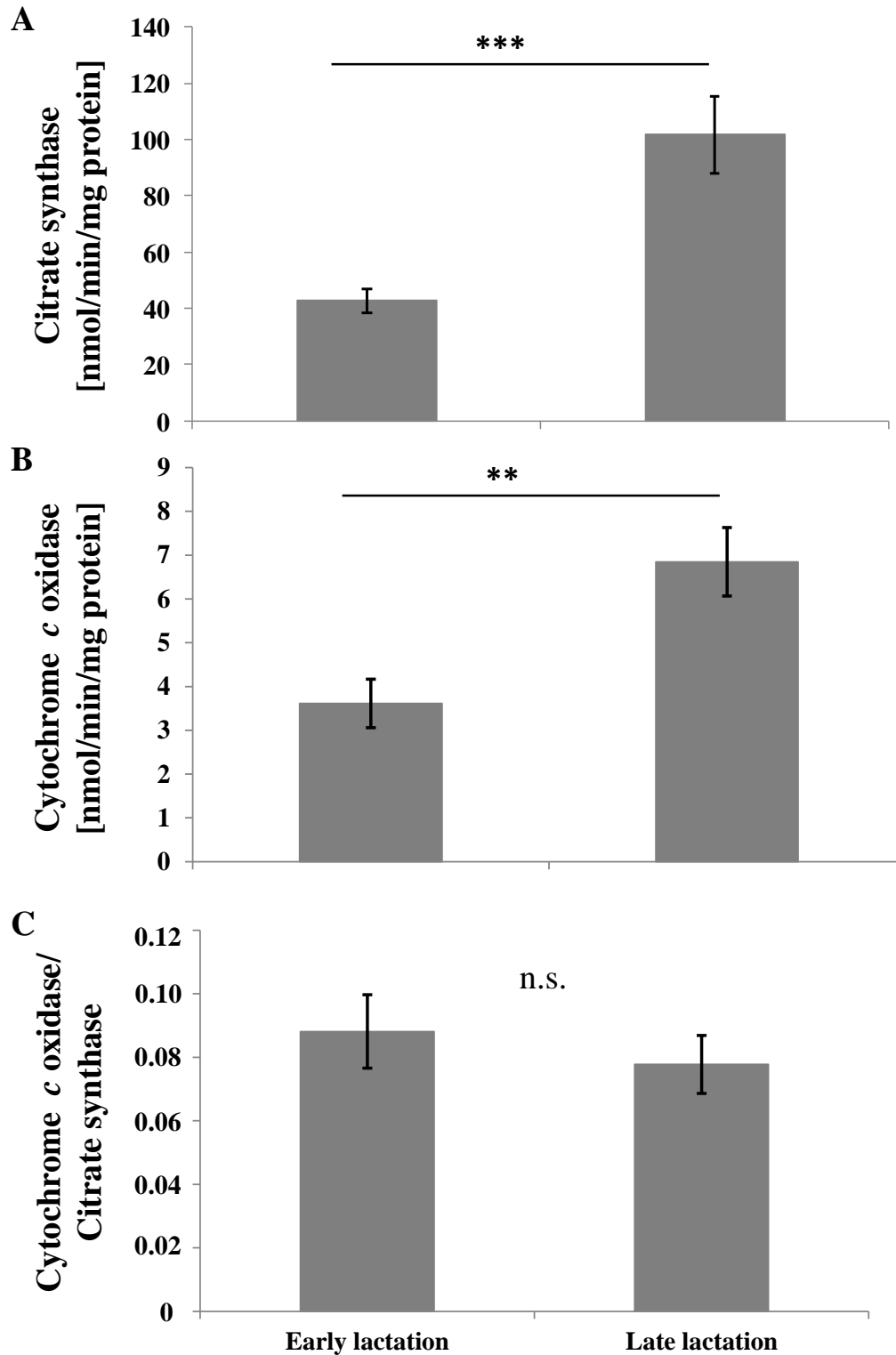
Figure 3 Activity of the mitochondrial enzymes citrate synthase (A; nmol/min/mg protein), cytochrome c oxidase (B; nmol/min/mg protein) and the ration between cytochrome c oxidase and citrate synthase (C) in adipose tissue from high-yielding dairy cows (n = 21) in early (21 to 28 DIM) and late (245 to 252 DIM) lactation. Data are presented as means ± SEM. ***: $P \le 0.001$, **: $P \le 0.01$, n.s.: not significant.

Tissue-specific and lactation driven changes of genes related to mitochondrial biogenesis

The mRNA abundances of key transcription factors of mitochondrial biogenesis (*NRF-1*, *NRF-2*, *TFAM* and *PGC-1α*) during early and late lactation in scAT, liver, and mammary gland are presented in Figure 4. The mRNA abundance of *NRF-1*, *NRF-2* and *TFAM* in liver remained constant, whereas *PGC-1α* mRNA abundance decreased at 25% (P =0.017; Fig. 4A) from early to late lactation. Within the scAT, the mRNA abundance of *NRF-2* and *TFAM* increased 1.2-fold (P =0.036) and 1.4-fold (P =0.004), respectively, whereas the *NRF-1* expression decreased at 11% from early to late lactation (P =0.022; Fig. 4B). The mRNA abundance of *PGC-1α* in mammary gland increased 1.3-fold from early to late lactation (P =0.006, Fig. 4C).
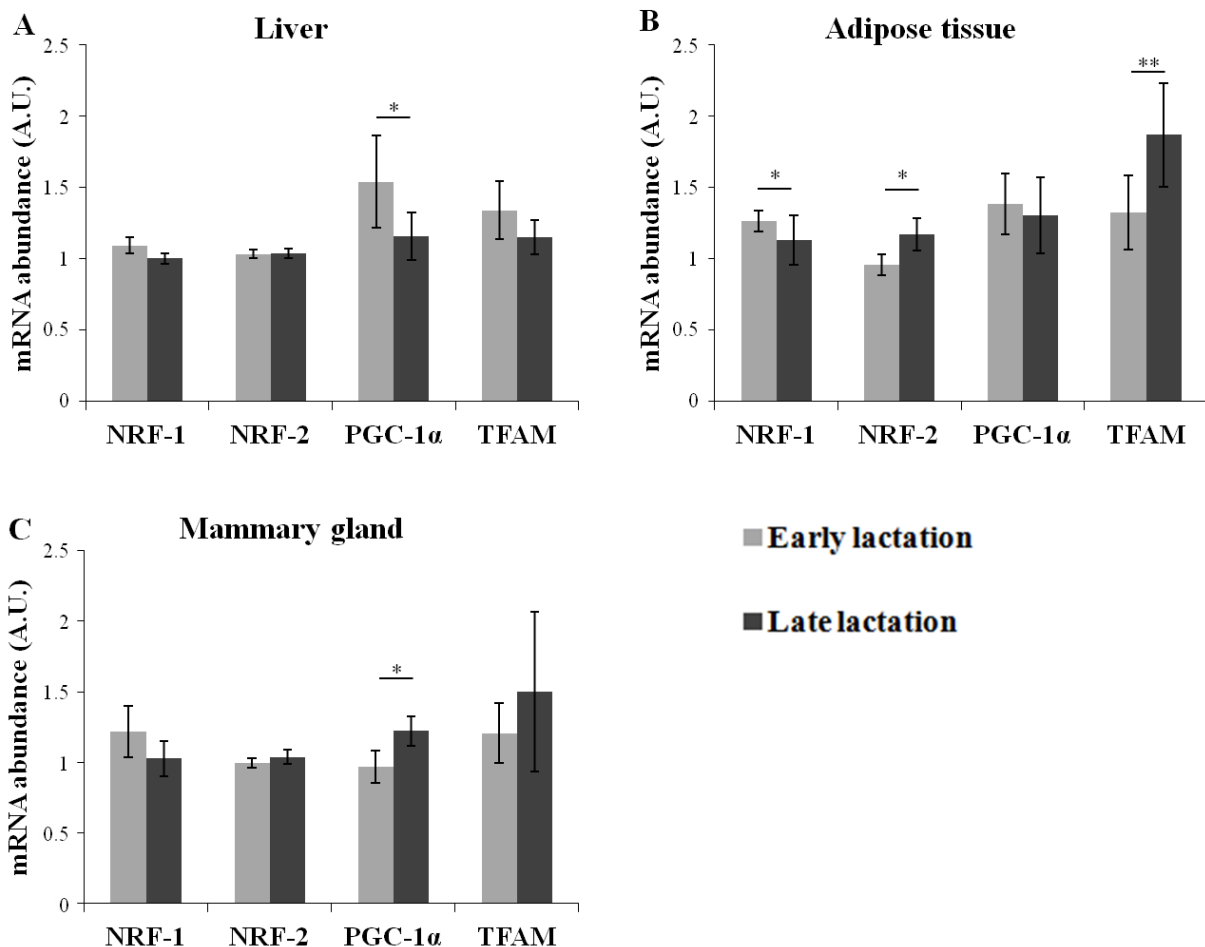
Figure 4 mRNA abundance (geometric means ± SEM) of genes related to mitochondrial biogenesis, *NRF-1*, *NRF-2*, *PGC-1α* and *TFAM*, in early and in late lactation in liver (A; n = 21), adipose tissue (B; n = 21), and mammary gland (C; n = 15) biopsies. Data are normalized based on the geometric mean of the selected most stable reference genes. Asterisks indicate significant differences between early and late lactation. *: P < 0.05; **: P ≤ 0.005.

Correlations between the mRNA abundance of mitochondrial biogenesis genes and mtDNA copy number

The correlations between mtDNA copy numbers and mRNA abundances of *NRF-1*, *NRF-2*, *PGC-1α* and *TFAM* in different tissues are shown in Table 3.

Table 3 Spearman correlation coefficients between mitochondrial DNA (mtDNA) copy number per cell and gene expression of mitochondrial biogenesis parameters in adipose tissue, liver, and mammary gland of lactating dairy cows.

		NRF-1	*NRF-2*	*PGC-1α*	*TFAM*
Adipose tissue	**mtDNA copy number**	n.s.	**0.340**	n.s.	**0.388**
	NRF-1	--	**0.486**	**0.601**	**0.424**
	NRF-2	**0.486**	--	**0.487**	**0.954**
	PGC-1α	**0.601**	**0.487**	--	**0.555**
Liver	**mtDNA copy number**	**0.515**	n.s.	n.s.	n.s.
	NRF-1	--	**0.370**	n.s.	n.s.
	NRF-2	**0.370**	--	n.s.	**0.384**
	PGC-1α	n.s.	n.s.	--	**-0.349**
Mammary gland	**mtDNA copy number**	n.s.	n.s.	n.s.	n.s.
	NRF-1	--	n.s.	**-0.390**	**0.769**
	NRF-2	n.s.	--	*0.323*	n.s.
	PGC-1α	**-0.390**	*0.323*	--	**-0.352**

Bold: $P \leq 0.05$; italic: $0.05 < P \leq 0.1$; n.s.: not significant

BCS was positively correlated with scAT mtDNA copy number ($\rho = 0.322$; $P = 0.038$) and negatively correlated with blood mtDNA copy number ($\rho = -0.420$; $P = 0.006$).

The mtDNA copies in peripheral blood were weakly correlated with mtDNA in liver ($\rho = 0.343$; $P = 0.026$); however, no relation was observed with mtDNA of scAT and mammary gland. In addition, mtDNA copies of peripheral blood showed a moderate positive correlation with milk yield ($\rho = 0.420$; $P = 0.006$), and a weak negative relation to energy balance ($\rho = -0.355$; $P = 0.023$). NEFA concentrations were negatively correlated to mtDNA copies in liver ($\rho = -0.421$; $P = 0.006$) and scAT ($\rho = -0.418$; $P = 0.007$).

In early lactation, peripheral blood mtDNA copy numbers were strongly correlated with mtDNA copies in liver (ρ =0.727; P <0.001), scAT (ρ =0.762; P <0.001), and mammary gland (ρ = 0.711; P =0.001). However, no relationship was observed between these variables in late lactation (see Figure 5).
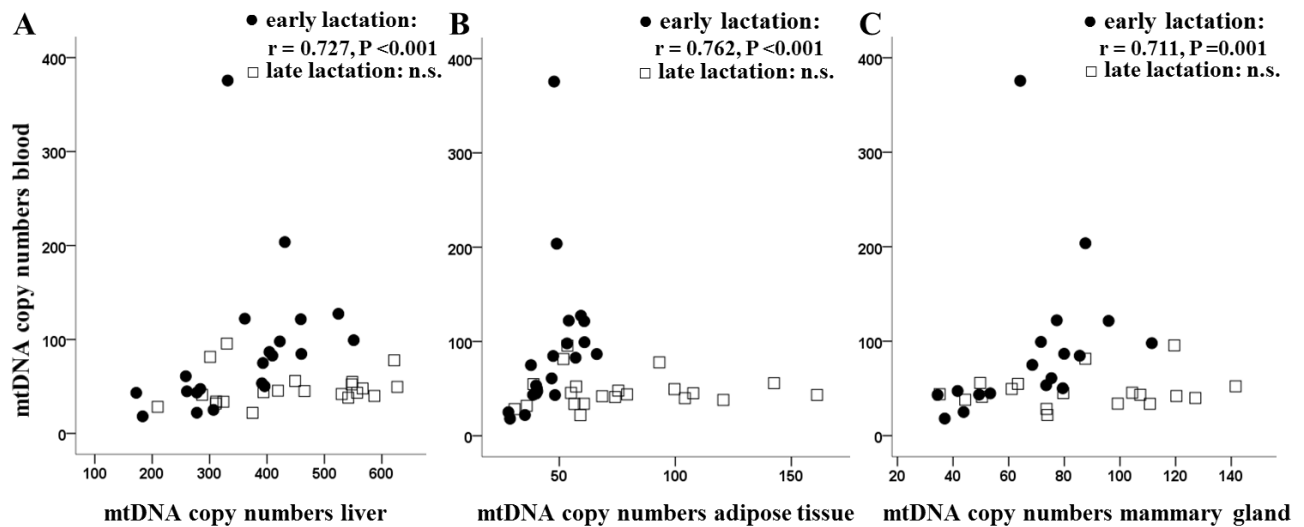
Figure 5 Relationships between mitochondrial DNA (mtDNA) copy numbers in peripheral blood and liver (A; n = 21), adipose tissue (B; n = 21) as well as mammary gland (C; n = 19) in high-yielding dairy cows from early (●; 21 to 28 DIM) and late (□; 245 to 252 DIM) lactation. P ≤0.05; n.s.: not significant

DISCUSSION

With the onset of lactation, the energy requirement rapidly increases in high-yielding dairy cows due to milk production; voluntary feed intake is usually not increasing as fast as does milk energy output. The adaption to lactation requires an elaborate regulation and coordination of energy metabolism among the key organs, such as AT, liver, and mammary gland (Barber et al., 1997; Block et al., 2001). Mitochondria are the main source for energy production in mammalian cells and the mtDNA copy number, which reflects the abundance of mitochondria in a cell, can adapt to the energy demand and physiological condition of each individual (Al-Kafaji and Golbahar, 2013). Although mitochondria ubiquitary occur in each cell, mitochondrial content, activity, and biogenesis can vary in different cell types in response to metabolic regulation (Lee et al. 2014). Mitochondrial membranes can be damaged by excessive triacylglycerol supply (Contreras and

Sordillo, 2011), which is of particular interest in dairy cows, because of their dependence on fatty acids as a major energy source in the early postpartum period (Bauman and Currie, 1980). In mid and late lactation, energy for milk synthesis is declining; however, the energy demands in high-yielding dairy cows are still high, because of the needs for gestation, maintenance of lactation, and formation of body reserves for the next lactation period. An adequate population of healthy mitochondria is not only essential for cell survival, but also guarantees the energy requirements for metabolically active organs throughout the whole lactation in dairy cows. In order to elucidate the cellular energy status in high-yielding dairy cows, we aimed to analyze the mtDNA copy numbers and the mRNA expression of the main transcriptional regulators being involved in mitochondrial biogenesis in peripheral blood, scAT, liver, and mammary gland during early and late lactation.

Tissue-specific differences of mtDNA copy numbers in liver, mammary gland, and scAT

The amount of mtDNA per cell differs widely among different cell types in rats and mice (Wellings et al., 1960). In the present study, the highest number of mtDNA copies was observed in liver, in which the calculated mean mtDNA content of approximately 400 copies per cell corresponded well to values reported for rats using the same quantification method (Nicklas et al., 2004). Multiple metabolic processes, i.e. glucose, lipid and protein metabolism and ketogenesis increase the energy demands in bovine liver (Baldwin, 1995), that can further stimulate mtDNA content. In the present study, general metabolic parameters such as albumin, total protein, and glucose concentrations remained stable from early to late lactation, whereas indicators for lipid metabolism, i.e. cholesterol and triglygerides were increased, which might be interpreted as an up-regulation of the lipid metabolism in late lactation in order to provide substrates for lipogenesis. In the adaptation of these metabolic processes, the liver plays a central role and an increased activity of liver enzymes such as ASAT, GLDH, and GGT can indicate for metabolic stress and liver lesions (Giannini et al., 2005). Therefore, increased GLDH and GGT concentrations in late lactation, which are beyond critical thresholds symptomatic for diseases, might indicate for a generally stimulated metabolism. Decreased numbers of hepatic mitochondria in cows during negative EB, concomitant with decreased ATP content were observed earlier (Baird, 1980). This lack leads to less available energy for metabolic processes in the liver, e.g. for protein synthesis, as described by Baird et al. (1980). In cows, increased mitotic activity of hepatocytes after calving (Reid and Collins, 1980) is followed by hepatocyte

hypertrophy in mid lactation (Reid et al., 1980). However, in the current study, hepatocyte area remained stable when comparing biopsies from early and late lactation. Based on the increased activity of liver enzymes, we suppose that the higher mtDNA copy numbers are rather related to the increased metabolic activity than to hypertrophy of hepatocytes.

Together with the calculated negative EB, increased lipolysis as indicated by NEFA concentrations > 0.2 mmol/L (Drackley, 2000), displays the high energy requirements in early lactation. Indeed, increased mtDNA copy numbers were observed in peripheral blood in early lactation as compared to late lactation. However, the number of mtDNA copies in liver was increased in late lactation and negatively correlated to NEFA concentrations. Based on the negative relationship between the circulating NEFA concentrations and the mtDNA copy number in liver and scAT, we suggest that elevated NEFA concentrations may down-regulate the number of mtDNA, since NEFA increase reactive oxygen species and are known to damage mitochondrial proteins and mtDNA (Wathes et al. 2012). High NEFA concentrations following a high-fat feeding were reported to reduce the mtDNA content in AT of rats (Sutherland et al., 2008). In humans, the accumulation of free fatty acids in visceral AT increased the synthesis of toxic fatty-acid-delivered metabolites and thus elevated the level of oxidative stress resulting in mitochondrial dysfunction accompanied by decreased mtDNA copy numbers (Lee et al., 2014). High-yielding dairy cows use NEFA released from AT as the major energy source, when the glucose supply is limited right after calving (Bauman and Currie, 1980). This may predispose towards mitochondrial damage in a variety of tissues (Wathes et al. 2012), because genes located in the mitochondrial matrix are upregulated and promote the use of NEFA for energy production via the electron transport chain, resulting in increased production of reactive oxygen species (Wathes et al. 2012).

In order to determine mitochondrial activity in AT, we analyzed the activity of CS and COX. Within the Krebs tricarboxylic acid cycle, CS is the rate limiting enzyme and has often been used as a mitochondrial marker (Holloszy et al 1970; Williams et al 1986). Proliferation of mitochondria can be associated with increased CS activity (Eigentler et al., 2015). One of the complexes in the oxidative phosphorylation system is COX (complex IV); the enzyme is located in the inner mitochondrial membrane and released in conjunction with mitochondrial damage (Renner et al. 2003). In the current study, both enzymes were increased from early to late lactation, supporting our findings of increased mtDNA content in AT. However, the COX/CS

ration, which is supposed to be a marker for mitochondrial function (Forini et al., 2012) remained constant throughout lactation.

Lipogenesis is an energy-consuming process, in which mitochondria must generate and provide sufficient ATP (Lu et al., 2010). In human white adipocytes, the mtDNA content was strongly positively related to lipogenesis (Kaaman et al., 2007). Given that AT depots are re-filled via lipogenesis during late lactation in dairy cows, the positive relation between BCS and mtDNA copy number in scAT might result from the lipogenic activity of AT. Recently, we found the same association between increasing BCS concomitant with increasing mtDNA copy numbers in AT of non-lactating cows after a diet-induced over-condition (Laubenthal et al., 2015).

Milk production highly depends upon mitochondrial ATP synthesis (Huang and Keenan, 1971). In humans and mice, the number of mitochondria increases in order to support lactogenesis during late pregnancy as well as the early postpartum period (until 8 days postpartum) and decreases thereafter (Wellings et al., 1960; Hollman, 1974; Rosano and Jones, 1976). Recently, Alex et al. (2015) observed increased mitochondria per mammary epithelia cells in early lactation and along with increased milk frequency in high-yielding dairy cows. Moreover, the number of mitochondria decreased from 15 DIM until 230 DIM (Alex et al. 2015). However, in the present study, mtDNA copies tended to increase when comparing early (21-28 DIM) and late (245-252 DIM) lactation. Our results were in accordance with the number of mitochondria in the mammary gland of lactating Chinese Holstein cows, showing increased mitochondria from late pregnancy to late lactation (280 DIM) with the highest values after 60 DIM (Qu et al., 2012). During the course of lactation, the ATP concentrations in mammary gland alter in cows, with greatest values during periods of peak milk production (Waldschmidt, 1973). Thus, the increasing numbers of mtDNA copies observed in the current study might expand the capacity for ATP synthesis within the mammary cells.

Gene expression of regulators of mtDNA transcription

In the lactation cycle, changes of mitochondrial function and biogenesis were related to milk production in mice (Hadsell et al. 2011). However, the regulation of mitochondrial biogenesis and function in dairy cows is poorly understood. Tissue-specific mtDNA copy numbers imply a tissue-specific expression of genes involved in maintenance and regulation of mtDNA. Therefore, the expression of the genes encoding *TFAM*, *PGC-1α*, *NRF-1* and *NRF-2* was determined herein. Besides its role in mtDNA transcription, initiation, and mtDNA replication (Shadel and Clayton,

1993), *TFAM* is also important in mtDNA maintenance as a major component of the nucleoid (Kang et al., 2007). The results in scAT suggests, that up-regulation of *TFAM* might precede increasing mtDNA contents and thus enhances mtDNA transcription as it was shown for humans (Pejznochova et al., 2010).

The expression of *TFAM* in turn is regulated by *NRF-1* and *NRF-2*, while *NRF-1* and *NRF-2* mRNA abundance are affected by *PGC-1α* (Virbasius and Scarpulla, 1994; Wu et al., 1999). The varying gene expressions in different tissues observed in this study are in compliance with findings from Pejznochova et al. (2010). Mitochondrial transcription factors seemed to be expressed and/or regulated in a tissue-specific manner during lactation. Furthermore, Pejznochova et al. (2010) indicated a transcriptional regulation of mitochondrial proliferation in liver, whereas in muscle, mitochondrial biogenesis could rather be regulated on post-transcriptional or translational level. However, due to the lack of relationships between transcriptional factors and mtDNA copy numbers in mammary gland and liver, transcriptional regulation of mitochondrial proliferation seems to be limited to scAT in dairy cows.

Blood mtDNA as an indicator for tissue mtDNA content in dairy cows

Given that blood cells circulate in the whole body and organs, the mtDNA copy number in peripheral blood cells may reflect the mtDNA content in the investigated tissues. The weak correlation between peripheral blood mtDNA copy numbers and liver mtDNA copy numbers is in accordance with experiments in rats, where changes in mtDNA content of peripheral blood leukocytes were accompanied by similar changes in hepatocytes (Chen et al., 2012). However, the lack of correlation between mtDNA copies in blood and scAT or mammary gland, is in line with a human study on metabolic syndrome patients, in which reduced mtDNA copies in peripheral blood were not related to mtDNA content in white adipocytes (Mozhey et al., 2014). Thus, peripheral blood mtDNA copy numbers might not serve as an appropriate marker for tissue mtDNA content in dairy cows during the entire lactation. However, strong relationships were observed between mtDNA copy number in blood and tissues when limiting the comparison to early lactation. In humans, peripheral blood mononuclear cells have been used as surrogate markers for changes in muscle and brain, because their expression levels can change according to physiological and environmental events (Rudkowska et al., 2011; Lunnon et al., 2012). Therefore, we suppose that in early-lactating dairy cows, mtDNA copy numbers in blood might

reflect the current cellular energy status of scAT, liver, and mammary gland and may thus substitute tissue biopsies, which are more difficult to access.

CONCLUSIONS

In summary, the metabolic activity of the entire organism as well as of single tissues and cells can vary during early and late lactation. Highest mtDNA copy numbers in liver compared to all other tissues and blood, support the central metabolic role of this organ throughout the whole lactation. In dairy cows, both, the onset of lactation and the maintenance of lactation together with gestation and formation of body reserves for the next lactation influence cellular energy requirements, which was reflected in increasing mtDNA copy numbers in all tissues. In early-lactating dairy cows, blood mtDNA copy numbers may serve as a surrogate marker for the cellular energy status of tissues, in case tissue biopsies are difficult to assess. The association between the mtDNA copy numbers and transcriptional factors during lactation was limited to scAT and mitochondrial biogenesis seems to be regulated in a tissue-specific manner. However, further studies on the role of mitochondrial content, biogenesis and function are needed, in order to understand the complex mechanisms of cellular energy supply in dairy cows.

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5   Manuscript III (submitted):

Short communication: **Telomere lengths in different tissues of dairy cows during early and late lactation**

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INTERPRETIVE SUMMARY

Telomere lengths in different tissues of dairy cows during early and late lactation. *Laubenthal et al.* In view of reports about accelerated telomere shortening in stressful conditions in humans, we aimed to investigate telomere length during early lactation – the most challenging time for dairy cows – and late lactation. Both, parturition and lactation may provoke metabolic stress that may reduce the productive lifespan in dairy cows. Comparing blood cells and physiologically relevant tissues with different cellular turn-over rates, we found that telomere lengths varied in blood and different tissues and were affected by lactation.

ABSTRACT

The energy requirement in dairy cows change substantially within the last weeks before parturition and the first weeks of lactation. Metabolic key organs, in particular the mammary gland, adipose tissue (AT), and the liver are involved in regulating and coordinating the energetic processes during this stressful period. Chronic stress in dairy cows is accompanied by an increased susceptibility to metabolic and infectious disorders. Furthermore, in high-yielding dairy cows, metabolic stress due to lactation may result in a reduced productive lifespan. Longevity and chronic stress in humans can knowingly be determined by the quantification of telomere length (TL). Capping the ends of the chromosomes, telomeres shorten with every cell division and their attrition is influenced by stress-related conditions.

In order to provide an overview of TL in liver, subcutaneous (sc) AT, mammary gland, and peripheral blood cells during early and late lactation, we investigated primi- (PP) and multiparous (MP) German Holstein cows (n=21). Animals were fed according to their requirement and biopsies from scAT, liver, mammary gland as well as blood cells were collected in early and late lactation. The relative quantity of telomere products (qT) which is proportional to the average TL, was determined in genomic DNA by multiplex qPCR.

In this study, relative qT varied widely in the investigated tissues and blood. In late lactation, poorly proliferating tissues, such as liver and scAT had the highest qT, whereas qT was lowest in peripheral blood cells and in the mammary gland. Comparing early to late lactation, relative qT reduction was limited to blood and mammary gland. We did not observe any difference in qT between PP and MP cows. Cows with high initial qT in tissues and blood in early lactation had greater qT reduction during the course of lactation than cows with lower qT. Measurement of relative qT should thus be included in phenotyping dairy cattle in order to test for associations with performance and fitness traits.

Keywords: Dairy cow, Lactation, Telomere length

SHORT COMMUNICATION

The first weeks of lactation in dairy cows are characterized by a negative energy balance (**EB**) in consequence of a marked increase in milk production coming along with reduced feed intake (Rukkwamsuk et al., 1999). This energy deficit is primarily compensated by mobilization of body reserves mainly from adipose tissue ((**AT**); McNamara, 1989). In the course of lactation when EB reaches positive values, fat depots are re-filled by lipogenic processes. Stressful events such as parturition and lactation substantially change the metabolic activity of key organs, particularly the mammary gland, AT, and liver in dairy cows (Barber et al., 1997; Block et al., 2001). Dairy cows being exposed to chronic stress are more susceptible to metabolic and infectious diseases (Broom and Fraser, 2007) and often show reduced fertility (Sapolsky et al., 2000). Furthermore, metabolic stress and reduced fertility are associated with reduced productive lifespan in dairy cows (Pritchard et al., 2013; Wathes, 2012).

In humans, the telomere length (**TL**) serves as a biomarker for cellular and biological aging (von Zglinicki and Martin-Ruiz, 2005), chronic stress (Epel et al., 2004), and longevity (Bakaysa et al., 2007). Telomeres are repetitive DNA sequences (TTAGGG) capping the end of the chromosomes, to protect them against degradation and fusion (Blackburn, 1991). Telomeres shorten with every cell division (Harley et al., 1990) due to the inability of DNA polymerase to replicate the lagging strand of chromosomes (Blackburn, 1991; von Zglinicki and Martin-Ruiz, 2005). The enzyme telomerase can maintain TL by adding tandem repeats *de novo* to the ends of the chromosomes; however, its activity in somatic cells is too low to enable full maintenance of TL (von Zglinicki et al., 2000). When TL declines to a critical point, the telomeres become dysfunctional, leading to cellular replicative senescence followed by cell death (Armanios and Blackburn, 2012).

The TL is affected by genetics (Njajou et al., 2007), stress-related conditions, inflammation, oxidative stress, and environmental factors (Entringer et al., 2011). In human peripheral blood cells, TL slowly declines with increasing age (Slagboom et al., 1994). Recently Brown et al. (2012) demonstrated a relationship between peripheral blood TL and survival of Holstein dairy cows, indicating for a potential role of TL to assess stress and health conditions in cows. To our knowledge, no report exists comparing TL in different tissues and TL changes during early and

late lactation in dairy cows. Therefore, the present study aimed to investigate 1) TL in physiologically relevant tissues of lactating dairy cows, namely AT, liver, and mammary gland as well as in peripheral blood cells and 2) changes of TL between early and late lactation. Given that primiparous (**PP**) cows are more prone to increased stress levels and compromised welfare and production right after calving than multiparous (**MP**) cows (Gonzáles et al., 2003), we hypothesized that TL can be affected by parity. Therefore, we compared TL in different tissues and peripheral blood from PP and MP cows with regard to lactation number. Moreover, we tested the use of TL in peripheral blood as a potential surrogate marker for TL in tissues avoiding biopsies in dairy cows.

The animal trial was conducted at the experimental station Frankenforst of the Faculty of Agriculture, University of Bonn, Königswinter, Germany. Both PP (age: 2 years; n = 4) and MP (age: 3 - 6 years; n = 17) German Holstein cows were fed diets according to the recommendations of the Society of Nutrition Physiology in Germany (GfE, 2001) with a partial mixed ration (6.3 – 6.8 MJ NE_L/kg DM) offered for *ad libitum* intake and concentrate (7.7 MJ NE_L/kg DM) depending on the individual's milk yield. Animals were housed in a freestall barn with adjacent milking parlor and were milked twice per day. The net EB was calculated by subtracting the daily requirement for maintenance (GfE, 2001) and the daily requirement for milk production (Tyrrell and Reid, 1965) from the daily energy intake with fixed values for fat and protein content (4% and 3.4%, respectively). Body weight (kg), feed intake (kg), and milk yield (kg) were recorded daily and body condition scores [**BCS**, according to the 5-scale system by Edmonson et al. (1989)] were monitored monthly and on the day of the biopsy.

Biopsies from subcutaneous (**sc**) AT of the tailhead region, from liver, and from the mammary gland were taken in early (21 - 28 DIM) and late (245 - 252 DIM) lactation. All biopsy samples were rinsed in saline, immediately snap frozen in liquid nitrogen and stored at -80°C until further analyses. Blood samples were collected from the jugular vein prior to the biopsies and after centrifugation serum and heparin-plasma were stored at -80°C, respectively. The DNA was isolated from whole heparin-blood. BHBA and NEFA concentrations (mmol/L) were determined in serum by an automatic clinical chemistry analyzer (Eurolyser CCA180, Eurolab, Hallein, Austria).

The relative quantities of telomere products (**qT**), which strongly correlate with relative TL, were assessed after DNA isolation by a multiplex qPCR (Cawthon, 2009). Total genomic DNA from scAT biopsies was extracted using the PowerPlant® Pro DNA Isolation Kit (MOBIO, Carlsbad, CA) and DNA from whole blood, liver, and mammary gland tissue biopsies was isolated with the Wizard Genomic DNA Purification Kit (Promega, Mannheim, Germany). The concentration and purity of total DNA were assessed on a Nanodrop 1000 device (peQLab Biotechnology, Erlangen, Germany) at 260 nm and 280 nm. Gel electrophoresis was used to evaluate the integrity of the DNA. Ten ng/µL of total DNA were mixed with two sets of primers: one amplified telomeres, whereas the other one was specific to the bovine ß-globin gene, a housekeeping gene that operates as the nuclear control gene for determining the relative qT. Primer sequences and PCR conditions were performed as described previously including minor modifications of the thermal conditions (Brown et al., 2012). In brief, the specificity of both primers was tested by gel electrophoresis. For multiplex qPCR, 10 µL Dynamo SYBR Green (Thermo Scientific, Rockford, IL), 0.12 µL ROX as passive reference dye (Thermo Scientific, Rockford, IL), both primers (1 µL each) and nuclease free water (final volume of 20 µL) were mixed. All samples were run in triplicates, a DNA standard curve was used to estimate PCR efficiency for each qPCR run and a pooled DNA sample served as inter-run calibrator. The analysis of the relative quantity of the telomeres to ß-globin was calculated as follows: $qT = PCR$ efficieny $(E)^n$, with $n = Ct_{\beta Globin} - Ct_{Telomere}$.

Statistical analyses were performed using SPSS version 22.0 (SPSS Inc., Chicago, IL). Data for all variables were tested for normal distribution using the Kolmogorov-Smirnov test and for homogeneity of variances by the Levene's test. Data from early and late lactation were compared by using the pairwise Students' t-test and differences between tissues were analyzed using one-way ANOVA with Bonferroni Post Hoc Test. A mixed model was used to assess differences between animals of different parities with "lactation number" as fixed and "cow" as random effect with Bonferroni Post Hoc Test. All values are expressed as means ± SEM. Correlations were assessed by Pearson analysis. Results with a P-value ≤ 0.05 were considered significant and $0.05 < P \leq 0.1$ was set as a trend.

So far, data about TL in dairy cows are limited to blood measurements (Brown et al., 2012). Therefore, we aimed to investigate TL in different physiologically relevant tissues in

comparison to peripheral blood and to characterize potential changes in TL from early to late lactation in dairy cows.

Net EB, milk yield, body weight, BCS, NEFA, and BHBA concentrations in early and late lactation are given in Table 1.

Table 1 Body condition, performance, and blood variables of dairy cows (n = 21) during early (21 to 28 DIM) and late (245 to 252 DIM) lactation. Means ± SEM. $P \leq 0.05$.

	Lactation					
	Early			**Late**		
Energy balance (MJ NE_L/kg)	-6.89	±	3.83[a]	17.4	±	4.31[b]
Milk yield (kg)	33.2	±	1.72[a]	22.8	±	1.33[b]
Body weight (kg)	627	±	12.8[a]	657	±	14.1[b]
BCS	2.4	±	0.1[a]	3.0	±	0.1[b]
Blood variables						
NEFA (mmol/L)	0.49	±	0.04[a]	0.24[b]	±	0.02[b]
BHBA (mmol/L)	0.92	±	0.09[a]	0.57[b]	±	0.06[b]

Different letters indicate significant differences between early and late lactation. $P \leq 0.05$.

The relative qT in scAT, liver, mammary gland, and peripheral blood from early and late lactation are presented in Figure 1. Relative qT decreased by 28% ($P = 0.012$) in peripheral blood from early to late lactation. Similar changes of TL in blood cells within a time period of six months were observed in overweight humans (Svenson et al., 2011). Given that blood cells are fast replicating cells, determination of telomerase activity might be of particular interest, since tissues that renew throughout life may require a consistent regulation by telomerase (Wang et al., 2005).

In the mammary gland, relative qT decreased by 16% ($P = 0.02$) from early to late lactation and was positively correlated with milk yield (r = 0.421; $P = 0.01$) when taking both time points together. Increased cell proliferation in the mammary gland from MP cows is accompanied by decreased milk yield from early to late lactation (Capuco et al. 2001). Thus, high milk yield during early lactation might arise from an increased activity per each cell and reduction of

relative qT might be increased due to cell proliferation during later stages of lactation in the mammary gland.

Human TL are shorter in fast-regenerating than in slowly replicating tissues (Aubert and Lansdorp, 2008). In the present study, the lowest qT were observed in the mammary gland in early and late lactation. During the entire lactation, around 50% of mammary epithelial cells are renewed in dairy cows supporting milk synthesis and persistency of lactation (Capuco et al., 2001). Thus, low qT in the mammary gland might result from the high turnover rate of bovine mammary cells.
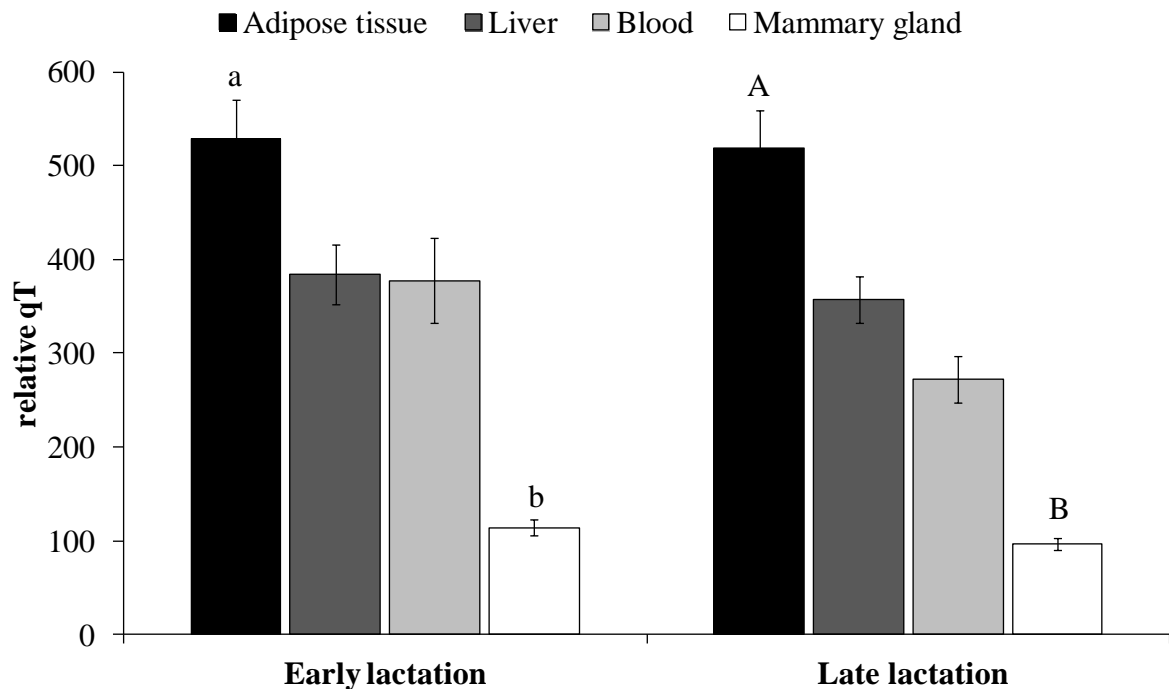
Figure 1 Relative quantities of telomere products (means ± SEM) in peripheral blood ($n = 21$), liver ($n = 21$), subcutaneous adipose tissue ($n = 21$), and mammary gland ($n = 19$) of primiparous and multiparous dairy cows during early (21 to 28 DIM) and late (245 to 252 DIM) lactation. Asteriks indicate for significant differences between early and late lactation; different letters specify the differences between the individual tissues within one sampling time. $P \leq 0.05$.

The relative qT in liver and in scAT did not change from early to late lactation. Both tissues belong to the category of low-proliferating tissues (Daniali et al., 2013). Moreover, cell

proliferation rates observed in scAT from early lactating cows were low (Häussler et al., 2013). In humans, only marginal changes of TL were observed during adulthood in tissues with low replication rates and marginal telomerase activity (Nussey et al., 2014). In addition, TL shortening might not be involved in the aging process of poorly proliferative tissues such as AT (Tzanetakou et al. 2012).

Moreover, in early lactation, the greatest qT were found in scAT with about 1.4-fold higher values compared to blood and liver and 4.6-fold higher values compared to mammary gland. In late lactation, relative qT in scAT were 1.9- fold higher than in blood, 1.5-fold higher than in liver and 5.4- fold higher than in the mammary gland (Fig 1).

Shorter TL in blood compared to AT were also observed in old humans and obese women (Butler et al., 1998; el Bouazzaoui et al., 2014). These results may account for a faster turnover of blood cells as compared to cells in AT (el Bouazzaoui et al., 2014).

Tissues and organs are composed of multiple cell types with different mitotic activity, functions and telomerase activity (Blouin et al., 1977); therefore, the rate of TL shortening can differ within tissues (Aubert and Lansdorp, 2008). In isolated human adipocytes, TL were significantly shorter than in the whole AT (el Bouazzaoui et al., 2014). Future investigations should thus include homogenous cell populations to exclude or reduce the influence of nonviable cells and cell-type mixtures on TL examinations as described by Thomas et al. (2008).

In the present study, differences in the amount of relative qT were neither observed between PP and MP cows nor between cows of different lactation numbers (data not shown). Inheritance is known to be a major determinant of TL (Svenson et al., 2011), however, no information concerning the initial TL at birth was available for the animals investigated herein. Based on the current results, reduction of relative qT in dairy cows seems to be rather influenced by the time point of lactation than by the lactation number.

The relationships between relative qT in blood, mammary gland, AT, and liver are shown in Table 2.

Table 2 Pearson's coefficients of correlation between the relative quantities of telomere products (qT) in peripheral blood, liver, adipose tissue, and in the mammary gland of lactating dairy cows. The corresponding P-values are provided in parentheses.

	Blood	Liver	Adipose tissue
Liver	0.582	---	
	(<0.001)		
Adipose tissue	0.586	0.871	---
	(<0.001)	(<0.001)	
Mammary gland	r = 0.387	0.576	0.423
	(0.02)	(<0.001)	(0.01)

Based on the relatively low associations of relative qT in the investigated tissues with blood qT, determination of peripheral blood qT in dairy cows is not an appropriate indicator for tissue-specific qT. However, even though relative qT differed between the investigated tissues, we observed a strong correlation between qT in liver and scAT and moderate correlations between qT in these two tissues and qT in the mammary gland. Cows with high relative qT in one tissue seem to be equipped with high relative qT in general. Similar results were reported for humans, in which TL in liver, heart, and kidneys were strongly correlated (Takubo et al., 2002) and TL in leukocytes, fat skin, and muscle were also strongly related (Daniali et al., 2013).

The decrease of relative qT from early to late lactation, was higher in blood than in scAT ($P = 0.071$), and mammary gland ($P = 0.084$). Strong positive correlations were observed between the initial qT in early lactation and the extent of qT reduction in peripheral blood (r = 0.846; $P < 0.001$), liver (r = 0.633; $P = 0.002$), and mammary gland (r = 0.724; $P = 0.001$); in addition, the initial qT tended to be related to the qT reduction in scAT (r = 0.422; $P = 0.057$). Thus, the initial qT was identified as the main factor influencing the rate of qT reduction in bovine tissues: the higher qT were at the first sampling in early lactation, the more they decreased in the course of lactation. Similar observations were made by several other researchers, concluding that the rate of TL attrition depends on the length of the initial telomeres (Ehrlenbach et al., 2009; Nordfjall et al., 2009). One reason for this relationship might be that telomerase acts preferentially on short telomeres (Ehrlenbach et al., 2009). Whether telomerase is active in the investigated tissues and blood cells and supports TL maintenance remains to be investigated.

In summary, relative qT and qT reduction are tissue-specific in dairy cows. Our results indicate that qT determinations should be included in phenotyping dairy cattle in order to test for associations with performance and fitness traits.

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6   General discussion and conclusions

Cellular energy supply via mitochondria during lactation

The present study was carried out to characterize mtDNA and genes related to mitochondrial biogenesis during early and late lactation in primiparous (PP) and multiparous (MP) cows. The copy number of mtDNA in dairy cows increased tissue-specific from early to late lactation in AT, mammary gland and liver. However, the mtDNA content in peripheral blood, accompanied by greater energy needs, was higher in early compared to late lactation. In rats, mtDNA increased in response to calorie restriction and thus prevented an age-related decline of mtDNA content (Picca et al., 2014). This may also count for bovine blood mtDNA during NEB, when feed intake is diminished concomitantly with the enhanced metabolic load. Furthermore, we compared PP and MP cows, since the cellular energy metabolism might differ between both groups. However, mtDNA content and mRNA abundance of genes related to mitochondrial biogenesis were alike in tissues and in blood of PP and MP cows. Thus, we conclude that parity does not influence mitochondrial dynamics; however, considering the small sample number within the PP group, further investigations including more animals need to be performed.

Mitochondrial proliferation in AT is mainly regulated on transcriptional level in dairy cows, as we observed a significant correlation between increasing mtDNA content and TFAM mRNA expression. TFAM is important in mtDNA maintenance as a main element of the nucleoid (Kang et al., 2007), and for mtDNA transcription and replication of the mitochondrial genome (Shadel and Clayton, 1993). Therefore, TFAM mRNA level might also be related to mtDNA content. Based on our results in AT, enhanced TFAM transcript might precede increasing mtDNA contents as it was shown for humans (Pejznochova et al., 2010). Furthermore, we found a significant interdependence of NRF-1, NRF-2, PGC-1α and TFAM only in AT, which indicate that mitochondrial biogenesis might be controlled at the post-transcriptional or the translational level rather than by transcriptional regulation in mammary gland and liver of dairy cows.

The precise mechanisms being responsible for the decrease of peripheral blood mtDNA and vice versa the increase in tissue mtDNA in late lactation remain to be investigated.

Tissue-specific mtDNA content during lactation

Size, shape, and abundance of mitochondria as well as the number of mtDNA copies widely differ among different cell types and tissues (Robin and Wong, 1988), and may change under varying energy requirements and physiological as well as environmental conditions (Lee and Wei, 2005). In the current study, the mtDNA copy number per cell was determined in AT, mammary gland, liver, and blood of lactating cows, to provide an overview about potential tissue-specific differences.

The greatest mtDNA content was found in liver, followed by the contents in mammary gland, peripheral blood, and AT. Tissue-specific differences of mtDNA content were observed earlier in humans between muscle and leucocytes (Hsieh et al., 2011) as well as between muscle and liver (Pejznochova et al., 2010). In both studies the results were explained with the different replicative capacity and metabolic roles of the tissues investigated.

In the present study, the highest number of mtDNA copies found in liver supports that this organ has to fulfill crucial metabolic and energy-consuming processes during lactation (Baldwin, 1995). The mitotic activity of hepatocytes is greater directly after calving compared to mid lactation (Reid and Collins, 1980). In humans, highly mitotic-active tissues show less mtDNA content than tissues with a lower cell replication rate (Hsieh et al., 2011). Moreover, NEB accompanied by high NEFA concentrations might promote the reduction of the mtDNA content, since in bovine hepatocytes the number of mitochondria declines at calving (Baird, 1980). Also in our study, decreased mtDNA copy numbers were accompanied by increased NEFA concentrations during the NEB in early lactation. The same association between increased free fatty acids (FFA) concentrations and decreased mtDNA content was observed in human visceral AT. The authors of this study suggest that elevated FFA concentrations might promote an increase in the synthesis of toxic fatty-acid-delivered metabolites, which enhance oxidative stress levels and thus induce mitochondrial dysfunction (Lee et al., 2014).

The relatively low copy number of mtDNA in AT was negatively correlated with the NEFA concentrations in non-lactating as well as lactating cows in our studies. Moreover, in both studies the mtDNA content ascended with increasing BCS. Dairy cows refill their AT depots by lipogenic processes in mid to late lactation. Mitochondria can provide key substrates being necessary to support lipogenesis during adipogenesis and thus play a crucial role in the

differentiation and maturation of adipocytes. The sustained synthesis of fatty acids is one of the most energy-consuming processes in the cells; therefore, generation of ATP by mitochondria must be sufficient for normal cell activity as well as for fatty acid synthesis (Goldman et al., 2011). Hence, the increasing mtDNA content might play also a role during lipogenesis in bovine AT as it is proposed for humans (Kaaman et al., 2007). However, the precise mechanism by which mtDNA content in WAT could influence lipogenesis rate remains to be elucidated. Furthermore, in humans mtDNA copy number has been found to be positively associated with overweight and BMI (Mengel-From et al., 2014).

The positive relationship between adipocyte sizes and mtDNA copy number observed in over-conditioned cows, suggests that enlarged cells require more mitochondria to guarantee sufficient energy supply for cell survival. However, as we did not observe the same association between cell size and mtDNA content in hepatocytes, emphasizes the potential necessity of mitochondrial energy supply for lipogenic activities in AT.

The number of mtDNA copies in the mammary gland tended to be lower in early compared to late lactation, even though milk yield was expectedly higher in early lactation. Given that ATP synthesis is known to increase in order to enhance milk production in early lactation, mitochondrial activity might be elevated instead of mitochondrial number (Waldschmidt, 1973) in the lactating mammary gland.

Effect of a diet-induced over-condition in non-lactating cows

Over-conditioning of dairy cows as it might happen in late lactation and the beginning of the dry period may result from problems in herd health or feeding management. In the present study, non-lactating, non-pregnant dairy cows served as an appropriate model to study the effects of over-conditioning on mtDNA and mitochondrial biogenesis independent from physiological changes related to parturition and lactation.

Markers for oxidative stress (dROM, TBARS) as well as mtDNA content were elevated with fat accumulation. Mitochondria are the main production site of ROS and also the immediate target of ROS attack (Nicholls et al., 2003). Thus, increased mtDNA content might serve as a compensatory mechanism to mtDNA damage, provoked by increased oxidative stress. Vice versa, increased numbers of mitochondria will promote the generation of more ROS. An increase

of mtDNA as a response to elevated oxidative stress has been observed earlier in human brain (Barrientos et al., 1997b), lung (Lee et al., 1998a), and muscle (Barrientos et al., 1997a). However, the gene expression of transcription factors for mitochondrial biogenesis, NRF-1, NRF-2 and TFAM, remained unchanged during the experimental period of 15 wks, whereas PGC-1α tended to rise with increasing body condition. Whether long term periods of high oxidative stress levels lead to changes of genes related to mitochondrial biogenesis in cows, as it is known for rats (Arduini et al., 2011), remains to be clarified.

Fat accumulation in AT was accompanied by an enlargement of adipocyte size. Expanded adipocytes require more mitochondria in order to meet the increased ATP demand of the larger cell (Yin et al., 2014). However, enlarged adipocytes are prone to hypoxia because angiogenic processes might impair the rapid remodeling of blood vessels, in order to ensure sufficient nutrient and oxygen supply (Pang et al., 2008). The adipocytes of the cows in our study showed increased expression of HIF-1α, the main marker for hypoxia, until week 8 of over-conditioning. The ascending mtDNA content might counterbalance this energy deficit temporarily (Carabelli et al., 2011), as evidenced by decreasing numbers of apoptotic cells in AT. Due to the hypoxic signal of the cells, angiogenic factors will be upregulated to enhance blood and nutrient supply in AT (Gorlach et al., 2001).

Cellular aging in dairy cows

Chromosome damages are associated with increasing age (Hastie et al., 1990). Telomeres are essential for chromosome stability; however, they shorten with every cell division. The rate of telomere shortening is influenced by environmental and genetic factors (Kappei and Londono-Vallejo, 2008). The metabolic and cardiovascular performance, which is required for the copious milk production of cows after calving, may be regarded as a very stressful situation for the animals. Given that stress is associated with accelerated aging in humans (Daubenmier et al., 2012), we speculated that in high-yielding dairy cows the cell turnover is enhanced, leading to an increased rate of telomere shortening and thus to accelerated aging. Therefore, we designed an experiment including PP and MP cows, with different numbers of parities, and collected samples from peripheral blood as well as from tissues, which are physiologically relevant, during early and late lactation.

Surprisingly we did not observe any differences in TL between PP and MP cows. These results suggest that the number of parities and thus the recurrent metabolic stress of lactation do not affect TL in dairy cows, as it is reported for humans. However, in our experiment we could not consider the effect of genotype, which is known to be a significant marker for TL (Svenson et al., 2011). Information regarding the initial TL at birth of the cows was not available. Therefore, it cannot be excluded that the PP cows in our study had shorter telomeres than the MP cows already during their fetal life.

Mitochondrial biogenesis might also play a role in aging, as mtDNA mutations and dysfunctions are associated with increasing age (Balaban et al., 2005; Wallace, 2005). Neither age- nor parity related differences in mtDNA content and expression of genes related to mitochondrial biogenesis were observed in the cows of our study. Whether mitochondrial activity or regulation of mitochondrial biogenesis is limited in older cows requires further investigation.

Tissue-specific TL-shortening during lactation

During lactation, TL varied among the investigated tissues; the shortest telomeres were found in the mammary gland, followed by telomeres in peripheral blood, liver and AT. Relative TL was significantly decreased in the mammary gland and in blood from early to late lactation, whereas AT and liver TL declined only numerically.

Telomeres are known to shorten with every cell division (Harley et al., 1990). Therefore telomere shortening in tissues with a fast-regenerating capacity is greater than in low-replicative ones (Aubert and Lansdorp, 2008). Around 50% of the mammary epithelial cells are renewed during one lactation cycle in dairy cows in order to increase milk synthesis and lactation-persistency (Capuco et al., 2001). The TL in the mammary gland was positively associated with milk yield; therefore, we suggest that the shortest telomeres found in this tissue compared to the other investigated tissues might result from its high cell turnover rates. Furthermore, the greatest rates of TL shortening from early to late lactation were found in the mammary gland and in blood. This might indicate the dependence of telomeres from the individual's replicative potential of tissues and cells. Our observations of nearly unaffected TL from early to late lactation in liver and AT might arise from the minimally proliferative capacity of cells belonging to these tissues (Daniali et al., 2013). However, it is of great interest to reveal the impact and activity of telomerase in the investigated tissues, as this enzyme is known to have the ability to maintain and

elongate telomeres (Shay and Bacchetti, 1997). Presumably, telomerase activity in AT and liver is higher than in mammary gland and blood, or it could be possible that telomerase acts overridingly in tissues of older cows. Thus, regarding the dynamics of TL in tissues of dairy cows during lactation, it is necessary to determine the impact of telomerase for understanding the precise mechanisms of tissue-specific differences in TL.

The aptitude of TL and mtDNA as biomarkers in dairy cows

Biomarkers to identify dairy cows at risk for production diseases during lactation and for decreased productive lifespan are required for selectively supporting such cows by management, feeding or adequate therapies and also for phenotyping cows for breeding purposes.

Whether an increased or vice versa a decrease in mtDNA content is accompanied by more/less mitochondrial activity, concerning the generation of ATP, requires further investigations. However, based on our results, we consider using mtDNA copy number in blood of dairy cows during early lactation as appropriate to characterize the energy status, in terms of mitochondria numbers, of physiologically relevant tissues during this challenging time.

The possible use of TL measurement as a marker for stress in animals during lactation, as it was assumed by other authors (Brown et al., 2012), requires further investigation in our view.

However, TL in early lactation was strongly associated with the extent of TL reduction in blood and tissues. This might indicate that the main factor influencing the rate of TL shortening in bovine tissues is the initial TL: the longer the telomeres were at the first sampling in early lactation, the faster they decreased in length in the course of lactation. A reason for this occurrence might be that telomerase act preferentially on short telomeres. To verify this assumption the determination of telomerase activity is indispensable. However, the approach of determining TL should be included in phenotyping dairy cattle in order to test for associations with performances and fitness traits and could accordingly, serve as basis for a "deep" phenotyping for breeding purposes aiming to improve longevity in dairy cows.

7   Summary

Energy balance and metabolic status of dairy cows markedly change during lactation and thus a continuous adaption and regulation of metabolic key organs, i.e. adipose tissue (AT), liver, and mammary gland, is necessary. When energy demands cannot be covered by feed intake, dairy cows mobilize body reserves mainly from AT. The concomitant changes of fat mobilization at calving and early lactation, and fat accretion in late lactation require changes in AT angiogenesis to accomplish nutrient and oxygen supply for adipocytes. The extent of fat mobilization is more pronounced in cows that are over-conditioned at calving; furthermore, those cows are also susceptible to oxidative stress and metabolic disorders. The main energy providers in mammalian cells are mitochondria. The copy number of their own genome, the mitochondrial DNA (mtDNA) can alter according to varying environmental, physiological, and energy conditions and thus possibly also due to the stage of lactation. Moreover, metabolic stress, a condition that might occur in dairy cows during lactation, has been associated with reduced productive lifespan in dairy cows. The determination of the length of telomeres, short repetitive DNA sequences at the end of each chromosome, has become a common biomarker for aging and longevity in humans. As telomere length (TL) shortening depends on genetic and environmental influences, determination of TL in dairy cows might be a useful tool for breeding purposes to improve resilience.

Therefore, the experiments conducted herein aimed 1) to evaluate oxidative stress levels and their impact on AT mitochondrial biogenesis and angiogenesis after a diet-induced over-condition in cows, 2) to investigate mtDNA content and mitochondrial biogenesis in blood and tissues during different stages of lactation, and 3) to characterize TL in blood and tissues during different stages of lactation.

In the first trial, we aimed to determine the impact of nutrition and oxidative stress on mitochondrial biogenesis and angiogenesis in dairy cows independently from physiological changes related to parturition or lactation. Therefore, non-lactating and non-pregnant German Holstein cows (n = 8; age: 4 – 6 years) were adapted from a low-caloric straw-based diet to a high energy diet (through a stepwise increase of the concentrate portion from 0 to 60% of daily dry matter intake within 6 wks. This ration was fed for further 9 wks. Blood samples were taken

monthly and biopsy samples from AT of the subcutaneous (sc) tailhead region were collected at the beginning of the trial, after 8 and 15 wks, respectively.

In a second trial the effects of lactational stage on mtDNA, mitochondrial biogenesis and TL in lactating dairy cows were investigated. Primiparous (PP; n = 4) and multiparous (MP; n = 17) German Holstein cows were fed according to their requirements. Blood and biopsy samples from the liver, mammary gland and scAT were collected in early lactation (between 21 and 28 DIM) and late lactation (between 245 and 252 DIM).

In both studies, indicators for oxidative stress were measured in serum by quantification of derivates of reactive oxygen metabolites (dROM), via measuring thiobarbituric acid reactive substances (TBARS) and through assessing the concentrations of advanced oxidation protein products (AOPP). The copy number of mtDNA and the relative quantity of telomere products (qT) were determined by a multiplex qPCR in DNA extracted from tissues and peripheral blood. The mRNA expression of genes related to mitochondrial biogenesis and angiogenesis was examined via qPCR. Angiogenesis and apoptosis in scAT were evaluated by histological techniques.

Within manuscript 1 we demonstrated that over-conditioning of dairy cows, as it might happen in late lactation and the beginning of the dry period, led to increased concentrations of markers for oxidative stress. Due to the rapid fat accumulation, enlarged adipocytes of scAT showed an increased abundance of mitochondria and concurrently decreased apoptosis. This might indicate an improved energy supply within the adipocytes in order to compensate the oxidative stress condition and the potentially resulting mtDNA damage. More mitochondria might generate more molecules associated with oxidative stress, but the key transcription factors of mitochondrial biogenesis were largely unaffected. Thus, increased oxidative stress did not impair mtDNA in scAT of dairy cows. Furthermore, enlarged adipocytes were accompanied by local AT hypoxia due to the inability of angiogenic factors to induce proper remodeling of blood vessels in the rapidly growing AT. However, after 8 wks, the animals seem to have adapted to the high-caloric diet, characterized by stable cellular energy supply, AT angiogenesis, and levels of oxidative stress.

The cellular energy metabolism was also studied in the mammary gland, liver, scAT, and in blood from lactating PP and MP dairy cows (manuscript 2). Irrespective of the tissue type and

parity number, the mtDNA content increased from early to late lactation, whereas the mtDNA copies decreased in peripheral blood. However, mtDNA content was tissue-specific, whereby mitochondria were most strongly represented in liver, supporting the crucial role and enormous metabolic activity of this organ. Regarding the abundance of genes related to mitochondrial biogenesis, we also observed a tissue-specific response to lactational influences, even though regulation of mtDNA at the transcriptional level was only present in AT.

As tissue function critically depends on energy metabolism, analyzing the mtDNA contents in peripheral blood cells might be an attractive option for the assessment of the cellular energy status, in view of mitochondria numbers, of tissues during early lactation.

Moreover, we evaluated the effect of lactation on cellular aging by examining the relative TL in the aforementioned tissues and blood in cows from the second trial (manuscript 3). The relative TL were shortest in the mammary gland and in blood and decreased alike from early to late lactation in these organs only. No differences were observed between animals of different ages and thus numbers of lactation in the present study. However, in view of the low animal number of the PP cows, further work should focus on parity-related changes in TL within larger animal numbers.

Besides providing information about longitudinal changes of TL and mtDNA content during the different stages of lactation for the first time, the present thesis contributes to improve the knowledge about mitochondria and telomeres in various tissues and blood in dairy cows. Moreover, the dissertation serves as a basis for further studies exploring the role and regulation of mitochondria and telomeres in various physiological conditions in cattle.

8 Zusammenfassung

Die Laktation führt bei hochleistenden Milchkühen zu drastischen Veränderungen der Energiebilanz und des Stoffwechsels. Daher ist es dringend erforderlich, dass sich die für die Laktation wichtigsten Organe, Fettgewebe, Leber und Milchdrüse, an die sich verändernde Situation kontinuierlich anpassen, bzw. reguliert werden. Übertrifft der Energiebedarf die Energieaufnahme durch das Futter mobilisieren Hochleistungskühe ihre Körperreserven, die hauptsächlich aus dem Fettgewebe stammen. Sowohl die Fettmobilisierung nach dem Kalben und in der Frühlaktation, als auch die Fetteinlagerung während der späteren Laktationsphasen und dem Beginn der Trockenstehzeit, führt zu Veränderung in der Angiogenese des Fettgewebes, um eine Versorgung der Adipozyten mit Sauerstoff und Nährstoffen zu gewährleisten. Das Ausmaß der Fettmobilisierung ist bei Kühen die zum Zeitpunkt der Kalbung überkonditioniert sind besonders groß. Überkonditionierte Milchkühe sind darüber hinaus anfälliger gegenüber oxidativem Stress und daraus resultierenden metabolischen Erkrankungen. Mitochondrien sind die hauptsächlichen Energielieferanten in Säugetierzellen, wobei die Kopienanzahl ihres eigenen Genoms, die mitochondriale DNA (mtDNA), sich an variierende physiologische, energetische und umweltbedingte Zustände anpassen kann und damit vermutlich auch während der Laktation. Metabolischer Stress, ein Zustand, der während der Laktation auftreten kann, ist verbunden mit einer Verkürzung der Nutzungsdauer von Hochleistungskühen. Ein in der Humanmedizin gängiger Biomarker für die Alterung bzw. Langlebigkeit is die Bestimmung der Telomerlänge (TL). Telomere sind kurze, sich wiederholende DNA Sequenzen an den Chromosomenenden dessen Verkürzung sowohl genetisch als auch umweltbedingt beeinflusst wird. Daher könnte die Ermittlung der TL in Milchkühen ein nützliches Instrument für die Zucht von langlebigen Kühen darstellen.

Folglich zielten die hier aufgeführten Studien darauf ab: 1) den oxidativen Status von Milchkühen nach einer Fütterungsinduzierten Überkonditionierung zu ermitteln und dessen Einfluss auf die mitochondriale Biogenese und Angiogenese des Fettgewebes zu untersuchen, 2) den Gehalt von mtDNA und die mitochondriale Biogenese in Blut und Geweben während verschiedener Laktationsstadien zu charakterisieren und 3) die TL im Blut und in Geweben während der Laktation zu bestimmen.

Innerhalb des ersten Versuches wurde der Einfluss der Fütterung und die Auswirkungen von oxidativem Stress auf die mitochondriale Biogenese und die Angiogenese in Milchkühen unabhängig von laktations- und trächtigkeitsbedingten physiologischen Veränderungen untersucht. Acht nicht-laktierende und nicht-tragende Deutsche Holstein Kühe (Alter: 4 -6 Jahre) wurden von einer auf Stroh-basierenden Ration stufenweise auf eine hoch energiereiche Ration (Korn-Gras-Silage, *ad libitum*) umgestellt. Innerhalb der ersten sechs Versuchswochen wurde der Konzentratanteil in dieser Ration von 0 auf 60% des Trockenmassegehaltes erhöht. Diese Fütterung wurde für weitere neun Wochen beibehalten. Den Tieren wurden monatlich Blutproben und ergänzend zu Versuchsbeginn, nach acht und nach 15 Wochen Fettgewebsproben aus dem subkutanen Schwanzfett entnommen.

In einem zweiten Versuch wurde der Einfluss des Laktationsstadiums auf den mtDNA-Gehalt, die mitochondriale Biogenese und die TL in laktierenden Milchkühen untersucht. Die Versuchstiergruppe bestand aus primiparen (PP; n = 4) und multiparen (MP; n = 17) Kühen der Rasse Deutsche Holstein, die entsprechend ihrem Bedarf gefüttert wurden. Während der Frühlaktation (zwischen 21 und 28 DIM) und während der Spätlaktation (zwischen 245 und 252 DIM) wurden den Tieren Blutproben sowie Biopsien aus der Leber, der Milchdrüse und dem subkutanen Fettgewebe entnommen.

Der Nachweis von oxidativen Stress wurde im Serum durch die Bestimmung der Konzentrationen von Derivaten reaktiver Sauerstoffspezies (dROM), durch die Messung von Thiobarbitursäure-reaktiver Substanzen (TBARS), die eine Lipidperoxidation widerspiegeln, und durch die Bestimmung oxidativer Proteinprodukte (AOPP) durchgeführt. Die mtDNA Kopienanzahl und die relative Anzahl an Telomerprodukten (qT) wurden mittels einer Multiplex-qPCR in der DNA von Blut und den verschiedenen Geweben bestimmt. Die mRNA-Expression von Genen, die für wichtige Faktoren der mitochondrialen Biogenese und der Angiogenese kodieren, wurden mittels qPCR gemessen. Die Angiogenese und Apoptose innerhalb des subkutanen Fettgewebes wurde histologisch untersucht.

Das erste Manuskript zeigte, dass eine Überkonditionierung von Milchkühen, wie es in der Spätlaktation und dem Beginn der Trockenstehphase vorkommen kann, zu einem Konzentrationsanstieg der untersuchten oxidativen Stressparameter führte. Die aufgrund der schnellen Fettanreicherung vergrößerten Adipozyten zeigten ein erhöhtes Mitochondrien-

aufkommen im subkutanen Fettgewebe. Dies könnte auf eine gesteigerte Energieversorgung innerhalb der Adipozyten hindeuten, um die Situation des erhöhten oxidativen Stresses und die daraus eventuell resultierenden Schäden der mtDNA zu kompensieren. Auch wenn ein Anstieg der Mitochondrienanzahl mit der Entstehung von weiteren reaktiven Sauerstoffverbindungen verbunden ist, waren nahezu keine Veränderungen in der Genexpression von wichtigen Faktoren der mitochondrialen Biogenese feststellbar. Daraus kann gefolgert werden, dass oxidativer Stress die mtDNA im Fettgewebe der Milchkühe nicht beschädigt. Durch die Vergrößerung der Adipozyten kam es zu einer lokalen Hypoxie im Fettgewebe der Tiere. Ein Kausalzusammen-hang könnte darin bestehen, dass die für die Ausbildung und Elongation von Blutgefäßen verantwortlichen Wachstumsfaktoren mit der Bereitstellung einer ausreichenden Blut- und Sauerstoffversorgung dem sich schnell vergrößernden Fettgewebe nicht folgen können. Nach acht Wochen schienen die Tiere jedoch an die hoch-energiereiche Ration adaptiert zu haben, da sich die zelluläre Energie- und angiogenetische Versorgung sowie der oxidative Status stabilisiert hatten.

Der zellulären Energiestoffwechsel wurde zudem in der Milchdrüse, dem Fettgewebe, der Leber und im Blut von laktierenden PP und MP Kühen untersucht (Manuskript 2). Der mtDNA-Gehalt hat sich unabhängig von der Laktationsnummer von der Früh- zur Spätlaktation in allen Geweben erhöht; konträr dazu war der mtDNA-Gehalt im Blut innerhalb der gleichen Zeitspanne reduziert. Der Gehalt an mtDNA und damit die Anzahl an Mitochondrien zeigte eine gewebsspezifische Verteilung mit dem größten Aufkommen in der Leber, was für die immense metabolische Aktivität und die Bedeutung dieses Organs während der Laktation spricht. Ebenfalls ließen die untersuchten Gentranskripte der mitochondrialen Biogenese eine gewebsspezifische Expression während der Laktation erkennen, wobei die Regulation der mtDNA, basierend auf den Ergebnissen auf transkriptioneller Ebene nur im Fettgewebe vorzufinden war. Die Bestimmung der mtDNA Kopienanzahl in zirkulierenden Blutzellen, einem leicht zugänglichen Medium, könnte für die Untersuchung des Energiestatus von Geweben, hinsichtlich der Anzahl Mitochondrien, während der Frühlaktation attraktiv sein.

Des Weiteren wurde der Einfluss des Laktationsstadiums auf die Zellalterung, speziell auf die TL, in den obengenannten Geweben und in Blut bei den Milchkühen des zweiten Versuches untersucht (Manuskript 3). Die kürzesten Telomere wurden in der Milchdrüse und im Blut gefunden; ausschließlich in diesen beiden Organen wurde auch eine Verkürzung der TL von der

Früh- zur Spätlaktation festgestellt. Die geringe Tieranzahl in der PP-Versuchtstiergruppe könnte Grund dafür sein, dass keine Unterschiede zwischen verschieden alten Tieren gefunden wurden. Daher sollten zukünftige Untersuchungen zur TL eine höhere Tieranzahl beinhalten, um mögliche paritätsbezogene Unterschiede spezifischer untersuchen zu können.

In der vorliegenden Dissertation wurden zum ersten Mal Veränderungen der TL und des mtDNA-Gehaltes über einen längeren Zeitraum hinweg bei Milchkühen untersucht und beschrieben. Dabei wurde der Einfluss der Laktation und verschiedener physiologischer Stadien (Überkonditionierung, Früh- und Spätlaktation) in unterschiedlichen Geweben sowie im Blut analysiert. Diese Arbeit bildet damit eine Basis für weitere Untersuchungen um die Bedeutung und die Regulation von Mitochondrien und Telomeren in verschiedenen physiologischen Stadien bei Hochleistungskühen zu ergründen.

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11 Publications and proceedings derived from this doctorate thesis

1. **L. Laubenthal,** M. Hoelker, J. Frahm, S. Dänicke, K. Gerlach, KH. Südekum, H. Sauerwein & S. Häussler (2015): *Telomere lengths in different tissues of dairy cows during early and late lactation.* Journal of Dairy Science, *submitted.*

2. **L. Laubenthal,** M. Hoelker, J. Frahm, S. Dänicke, K. Gerlach, KH. Südekum, H. Sauerwein & S. Häussler (2015): *Mitochondrial DNA copy number and biogenesis in different tissues of early- and late lactating dairy cows.* Journal of Dairy Science, *submitted.*

3. **Laubenthal L,** Locher L, Sultana N, Winkler J, Meyer U, Rehage J, Dänicke S, Sauerwein H & Häussler S (2015): *The impact of oxidative stress on adipose tissue angiogenesis and mitochondrial biogenesis in over-conditioned dairy cows.* The Veterinary Journal, *submitted.*

4. **Laubenthal L,** Hoelker M, Südekum KH, Sauerwein H & Häussler S (2015): *Mitochondrial DNA copy numbers in blood cells during early and late lactation in dairy cows.* Journal of Animal Science /Journal of Dairy Science.

5. **Laubenthal L,** Hoelker M, Südekum KH, Sauerwein H & Häussler S (2015): *Mitochondrial DNA copy number in liver, mammary gland and adipose tissue of early lactating dairy cows.* Journal of Animal Science /Journal of Dairy Science.

6. **Laubenthal L**, Locher L, Sultana N, Winkler J, Meyer U, Rehage J, Dänicke S, Sauerwein H & Häussler S (2015): *Relationship between circulating leptin concentrations and adipocyte mitochondria in nonlactating dairy cows during a course of overcondition.* Proceedings of the Society of Nutrition Physiology. Band 24.

7. Locher L, Häussler S**, Laubenthal L,** Singh SP**,** Winkler J, Kinoshita A, Kenéz Á, Rehage J, Huber K, Sauerwein H & Dänicke S (2015): *Impact of increasing body condition on key regulators of fat metabolism in subcutaneous adipose tissue depot and circulation of nonlactating dairy cows.* Journal of Dairy Science, 98, 1057-1068.

8. **Laubenthal L**, Häussler S, Locher L, Winkler J, Meyer U, Rehage J, Dänicke S & Sauerwein H (2014): *Effect of excessive fat accumulation on Vascular Endothelial Growth Factor (VEGF) and von Willebrand Factor (vWF) expression in adipose tissue of dairy cows.* Proceedings of the Society of Nutrition Physiology. Band 23, 42, Page 68.

9. **Laubenthal L**, Locher L, Winkler J, Meyer U, Rehage J, Dänicke S, Sauerwein H & Häussler S (2014): *Association between oxidative stress through excessive fat accumulation and the number of mitochondrial DNA copies in adipose tissue of dairy cows.* Journal of Animal Science, 92/Journal of Dairy Science, 97, Page 681

10. **Laubenthal L**, Locher L, Winkler J, Meyer U, Rehage J, Dänicke S, Sauerwein H & Häussler S (2014): *Telomere length shortening in response to an excessive fat accumulation in subcutaneous adipose tissue of dairy cows.* Journal of Animal Science, 92/Journal of Dairy Science, 97, Page 681

11. **Laubenthal L**, Locher L, Winkler J, Meyer U, Rehage J, Dänicke S, Sauerwein H & Häussler S (2014): *Telomere length in different visceral and subcutaneous adipose tissue depots of overconditioned cows.* Journal of Animal Science, 92/Journal of Dairy Science, 97, Page 682

12. **Laubenthal L**, Locher L, Winkler J, Meyer U, Rehage J, Dänicke S, Sauerwein H & Häussler S (2014): *Differences in mitochondrial DNA copy numbers in various subcutaneous and visceral fat depots of overconditioned cows.* Journal of Animal Science, 92/Journal of Dairy Science, 97, Page 708

13. **Laubenthal L**, Locher L, Winkler J, Meyer U, Rehage J, Dänicke S, Sauerwein H & Häussler S (2014): *Adipose tissue hypoxia is related to increased mtDNA copies and decreased VEGF-A in fat dairy cows.* 65th Annual Meeting of the European Federation of Animal Science. Copenhagen, Denmark.

14. Häussler S & **Laubenthal L** (2014): *Telomere length: any perspective as a biomarker for longevity in dairy cows?* Proceedings of the First DairyCare Conference 2014, Health and Welfare of Dairy Animals, COST FA1308, held in Copenhagen, August 22nd and 23rd 2014, 6.2, Page 24

15. Häussler S**, Laubenthal L**, Locher L, Winkler J, Meyer U, Rehage J, Dänicke S & Sauerwein H (2014): *Macrophage infiltration into subcutaneous adipose tissue in overconditioned cows after excessive fat accumulation.* Journal of Animal Science, 92/Journal of Dairy Science, 97, Page 684

16. Häussler S**,** Locher L, **Laubenthal L**, Singh SP**,** Meyer U, Rehage J, Dänicke S & Sauerwein H (2014): *Association between insulin signaling and oxidative stress in serum and subcutaneous adipose tissue of overconditioned cows*. Journal of Animal Science, 92/Journal of Dairy Science, 97, Page 710

17. Häussler S**,** Sing SP**, Laubenthal L**, Locher L, Winkler J, Meyer U, Rehage J, Dänicke S & Sauerwein H (2013): *Impact of increased oxidative stress through excessive accumulation of adipose tissue on circulating adiponectin concentrations in dairy cows*. Journal of Dairy Science, 96, E-Suppl. 1, T328, 114