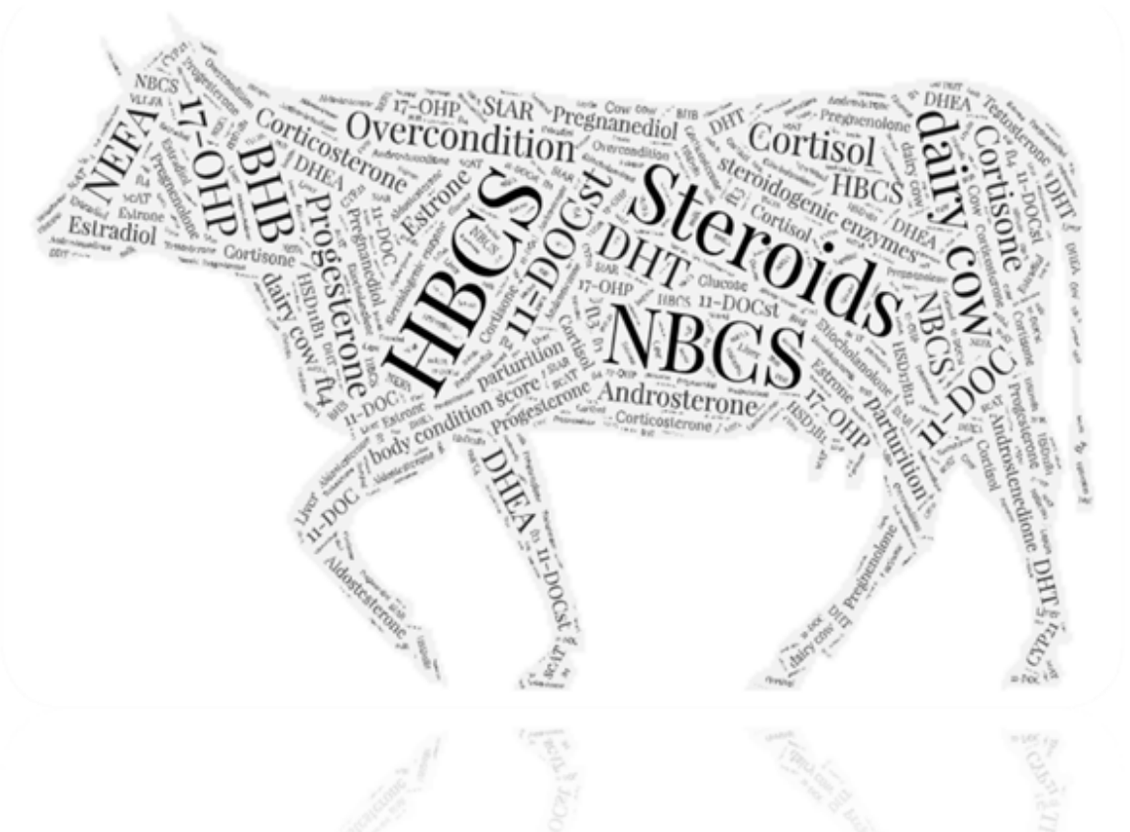
# Establishment of an experimental model for dairy cows of divergent peripartal mobilization of body reserves: Profiling circulating steroid hormones and the mRNA abundance of steroid metabolizing enzymes in liver and in adipose tissue

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Dissertation zur Erlangung des Grades
Doktorin der Agrarwissenschaften (Dr. agr.)
der Landwirtschaftlichen Fakultät
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von

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aus Trier

Bonn, 2021

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|---|---|
| Referentin: | Prof. Dr. Dr. Helga Sauerwein |
| Korreferenten: | Prof. Dr. Karl-Heinz Südekum |
| Tag der mündlichen Prüfung: | 13. August 2021 |

Angefertigt mit der Genehmigung der Landwirtschaftlichen Fakultät der Universität Bonn

## English abstract

The lactation cycle of dairy cows is characterized by physiological, metabolic, and endocrine changes that also affect adipose tissue (AT) function and portion. These changes include lipogenesis during periods of nutrient excess and lipolysis during periods of energy deficit. In addition to storing and providing energy, the AT is an important endocrine organ that is considered as a major reservoir of lipophilic steroid hormones which are locally converted by steroidogenic enzymes. Besides the naturally occurring mobilization of body reserves after parturition, cows that are over-conditioned before calving (a.p.) mobilize more fat after calving (p.p.) compared to normal conditioned cows along with an increased release of lipids from AT into the circulation. Consequently, stored steroid hormones may also enter the circulation. Steroids and steroidogenic enzymes have been described to contribute to obesity and local fat accumulation in humans, including a functional regulation of AT metabolism. To gain insights into how varying degrees of *post partum* lipolysis affects steroid metabolism in dairy cows, we established an animal model for comparing pluriparous cows with high (HBCS, N = 19) and normal body condition (NBCS, N = 19) a.p. Differences in body condition score (BCS) and backfat thickness (BFT) were maintained throughout the entire observation period from 7 weeks a.p. until 12 weeks p.p. The metabolic status was further characterized by assessing the energy status and milk yield together with the circulating concentrations of several metabolites and metabolic hormones. Here, the HBCS cows had consistently higher serum concentrations of fatty acids and of ß-hydroxybutyrate p.p., indicating higher body fat mobilization and ketogenesis compared to NBCS cows. For addressing the role of AT in steroid metabolism, the concentrations of 19 steroids in blood and 17 steroids in subcutaneous AT (scAT) were quantified using a targeted metabolomics approach. In addition, the mRNA abundance of five steroidogenic enzymes was assessed in liver and scAT samples collected in week -7, 1, 3, and 12 relative to calving. The concentrations of both scAT-accumulated and circulating steroids followed a comparable periparturient time course and were also influenced by parity. Increased lipolysis in HBCS cows p.p. was associated with greater concentrations of circulating androgens and progestins, which may reflect the release of these steroids from AT. Conversely, glucocorticoid concentrations were up to 3.5-fold higher in scAT of NBCS than in HBCS cows p.p.; however, the underlying mechanism of origin remains elusive and requires further validation.

The results of this work indicate that local steroid conversion in bovine AT is initiated by the steroidogenic enzymes steroid 21-hydroxylase and 17-ß-hydroxysteroid-dehydrogenase type 12 (HSD17B12). The increased hepatic mRNA abundance of HSD17B12 in HBCS relative to NBCS cows 7 weeks a.p. supports the notion that HSD17B12 may be involved in lipogenic processes. The present thesis provides first insights into the complex metabolism of endogenous steroid hormones in AT and blood of dairy cows differing in the intensity of lipomobilization after parturition, and provided a sound animal model for further research in this field.

## German abstract

Der Laktationszyklus von Milchkühen ist durch physiologische, metabolische und endokrine Veränderungen gekennzeichnet, die auch die Funktion und Anteil des Fettgewebes (AT) beeinflussen. Diese Veränderungen umfassen die Lipogenese in Zeiten eines Nährstoffüberschusses und die Lipolyse in Zeiten eines Energiedefizits. Neben der Speicherung und Bereitstellung von Energie ist das AT ein wichtiges endokrines Organ, das als wesentliches Reservoir für lipophile Steroidhormone angesehen wird, die lokal durch steroidogene Enzyme metabolisiert werden. Neben der natürlich vorkommenden Mobilisierung von Körperreserven nach der Geburt, weisen Kühe, die vor dem Kalben (a.p.) überkonditioniert waren nach der Kalbung (p.p.) eine erhöhte Fettmobilisierung im Vergleich zu normal konditionierten Kühen auf, einhergehend mit einer erhöhten Freisetzung von Lipiden aus dem AT in den Blutkreislauf. Folglich können auch gespeicherte Steroidhormone in den Blutkreislauf gelangen. Darüber hinaus wurde beschrieben, dass Steroide und steroidogene Enzyme bei humaner Adipositas und lokaler Fettansammlung eine entscheidende Rolle spielen, einschließlich der funktionellen Regulierung des AT-Stoffwechsels. Um Erkenntnisse darüber zu gewinnen, wie unterschiedliche Ausprägungen der postpartalen Lipolyse den Steroidstoffwechsel von Milchkühen beeinflussen, wurde ein Tiermodell etabliert, welches pluripare Kühe mit hoher (HBCS, N = 19) und mit normaler Körperkondition (NBCS, N = 19) a.p. vergleicht. Die Unterschiede in der Körperkondition (BCS) und der Rückenfettdicke (BFT) wurden über die gesamte Beobachtungszeit von sieben Wochen a.p. bis 12 Wochen p.p. aufrechterhalten. Die Stoffwechselsituation der Tiere wurde weiterhin durch den Energiestatus und die Milchleistung einhergehend mit den Gehalten verschiedener Metabolite und Stoffwechselhormone im Blut charakterisiert. Nach der Kalbung wiesen HBCS-Kühe höhere Serumkonzentrationen von Fettsäuren und ß-Hydroxybutyrat auf, welches auf eine höhere Körperfettmobilisierung und Ketogenese im Vergleich zu NBCS-Kühen hinweist. Um die Rolle des AT im Steroidmetabolismus zu untersuchen, wurden die Konzentrationen von 19 Steroiden im Blut und 17 Steroiden im subkutanem AT (scAT) mit einem gezielten Metabolomics-Ansatz quantifiziert. Zudem wurde die mRNA-Abundanz von fünf steroidogenen Enzymen in Leber und scAT Gewebeproben gemessen, die in den Wochen -7, 1, 3 und 12 relativ zum Kalben entnommen wurden. Die Konzentration AT-akkumulierter und zirkulierender Steroide zeigte einen vergleichbaren peripartalen Zeitverlauf, welche darüber hinaus von der Parität der Kühe beeinflusst wurde. Höhere Konzentrationen an zirkulierenden Androgenen und Gestagenen bei HBCS- versus NBCS-Kühen, wiesen auf eine vermehrte Freisetzung von Steroiden aus der erhöhten AT-Mobilisierung p.p. hin. Demgegenüber waren die Glukokortikoid-Konzentrationen im scAT von NBCS-Kühen p.p. bis zu 3,5-fach höher im Vergleich zu HBCS-Kühen; der zugrundeliegende Entstehungsmechanismus hierfür ist jedoch nicht eindeutig zu erklären und bedarf weitere Untersuchungen.

Die Ergebnisse dieser Arbeit deuten darauf hin, dass eine lokale Steroidkonversion im bovinen AT durch die steroidogenen Enzyme Steroid 21-Hydroxylase und 17-ß-Hydroxysteroid-Dehydrogenase Typ 12 (HSD17B12) initiiert wird. Die erhöhte mRNA-Abundanz von HSD17B12 in der Leber bei HBCS- im Vergleich zu NBCS-Kühen 7 Wochen a.p. unterstützt die Annahme, dass HSD17B12 an lipogenen Prozessen beteiligt sein könnte. Die vorliegende Dissertation gibt erste Einblicke in den komplexen Stoffwechsel endogener Steroidhormone im AT und Blut von Milchkühen, welcher sich in der Intensität der Fettmobilisierung nach der Kalbung unterscheidet und bietet mit dem etablierten Tiermodell eine solide Grundlage für weitere Forschung auf diesem Gebiet.

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## Abbreviations

| | |
|---|---|
| 11-DOC | 11-deoxycortisol |
| 11-DOCSt | 11-deoxycorticosterone |
| 17-OHP | 17-α-hydroxyprogesterone |
| AI | artificial insemination |
| AKRs | aldoketo reductases |
| a.p. | *ante partum* |
| AT | adipose tissue |
| BA | biogenic amines |
| BCS | body condition score |
| BCKDHA | branched-chain α-keto acid dehydrogenase E1α |
| BFT | backfat thickness |
| BHB | beta-hydroxybutyrate |
| BW | body weight |
| CI | calving interval |
| CK | clinical ketosis |
| CV | coefficient of variation |
| CYP11A1 | cholesterol monooxygenase (referred as: cholesterol side-chain cleavage enzyme; cytochrome P450scc) |
| CYP11B2 | aldosterone synthase (cytochrome P450aldo) |
| CYP17 | steroid 17α-hydroxylase (cytochrome P450c17) |
| CYP19 | aromatase (cytochrome P450arom) |
| CYP21 | steroid 21-hydroxylase (cytochrome P450c21) |
| DHEA | dehydroepiandrosterone |
| DHEA-S | dehydroepiandrosterone sulfate |
| DHT | dihydrotestosterone |
| DM | dry matter |
| DMI | dry matter intake |
| DP | dry period |
| dROM | derivatives of reactive oxygen metabolites |
| E1 | estrone |
| E1S | estrone-sulfate |

| | |
|---|---|
| E2 | estradiol-17ß |
| EB | energy balance |
| ECM | energy corrected milk |
| EIF3K | eukaryotic translation initiation factor 3, subunit K |
| ELISA | enzyme-linked immunosorbent assay |
| EMD | emerin |
| ER | endoplasmic reticulum |
| FI | feed intake |
| FRAP | ferric reducing ability |
| GC | Glucocorticoids |
| GfE | Gesellschaft für Ernährungsphysiologie |
| HBCS | high body condition group |
| Hp | haptoglobin |
| HPCAL1 | hippocalcin-like 1 |
| HSD | hydroxysteroid dehydrogenase |
| HSD3B1 | 3-ß-hydroxysteroid-dehydrogenase type 1 |
| HSD11B1 | 11-ß-hydroxysteroid-dehydrogenase type 1 |
| HSD17B12 | 17-ß-hydroxysteroid-dehydrogenase type 12 |
| IGF-1 | insulin-like growth factor 1 |
| IR | insulin resistance |
| IS | insulin sensitivity |
| LC-MS | liquid chromatography-mass spectrometry |
| LLOQ | lower limit of quantification |
| LOD | limit of detection |
| LRP10 | lipoprotein receptor-related protein 10 |
| MARVELD1 | marvel domain containing 1 |
| ME | metabolizable energy |
| MJ | mega joule |
| mRNA | messenger ribonucleic acid |
| miRNA | micro RNA |
| mTOR | mammalian target of rapamycin pathway |

## Abbreviations

| | |
|---|---|
| NBCS | normal body condition group |
| NEB | negative energy balance |
| NEFA | non-esterified fatty acids |
| $NE_L$ | net energy for lactation |
| OSI | Oxidative Stability Index |
| POL2 | RNA polymerase II |
| p.p. | *post partum* |
| qPCR | quantitative polymerase chain reaction |
| RQUICKI | revised quantitative insulin sensitivity check index |
| SEM | standard error of mean |
| scAT | subcutaneous adipose tissue |
| SCK | subclinical ketosis |
| SDRs | short-chain dehydrogenase/reductases |
| SR5A1 | steroid-5α-reductase |
| StAR | steroidogenic acute regulatory protein |
| STS | steroid sulfatase |
| T3 | triiodothyronine |
| T4 | thyroxine |
| TAG | triacylglycerols |
| TBF | total body fat |
| TBV | total blood volume |
| TG | triglycerides |
| TSPO | translocator protein |
| ULOQ | upper limit of quantification |
| UPS | ubiquitin-proteasome system |
| VLCFA | very-long-chain fatty acids |
| VLDL | very low density lipoprotein |

# 1 Introduction

High yielding dairy cows undergo tremendous metabolic changes along with physiological adaptations in order to maintain physiological equilibrium during the transition from late gestation to early lactation (Bauman and Currie 1980; Bell 1995). The management of the body condition score (BCS) in dairy herds is essential for maintaining healthy and productive cows. An optimal calving BCS (3.0 – 3.25; 5-point scale) plays a crucial role in terms of the extent of mobilizing energy stores and the sufficient nutrient intake after calving (Roche et al. 2009). Due to an augmented lipolytic activity, overconditioned cows (calving BCS > 3.75; 5-point scale) are more prone to experience excessive mobilization of body fat reserves than normal conditioned cows (de Koster et al. 2016). As a consequence, increased release of non-esterified fatty acids (NEFA) from adipose tissue break down and elevated production of ketone bodies (e.g., acetoacetate, acetone; due to incomplete NEFA oxidation (Herdt 2000)) may negatively affect immunity, fertility, milk yield, and welfare of the cows (Roche et al. 2009; Akbar et al. 2015).

The adipose tissue (AT) is an active endocrine organ converting and secreting metabolites, hormones, and cytokines alongside to lipophilic steroid hormones (Kershaw and Flier 2004). Besides their essential role in terms of sexual differentiation, reproduction, and fertility, sex-steroids have a huge metabolic importance in regulating lipolysis and AT deposition (Cooke and Naaz 2004). For a better understanding of the physiological importance of steroid hormones stored, released, or interconverted by the AT during the periparturient period of dairy cows, a more profound knowledge about the possible impact of differences in the extent of lipomobilization after parturition is required.

## *1.1 Homeorhetic adaptations during the periparturient period of dairy cows*

Decades of genetic selection aiming at high milk yield in dairy cows have resulted in amplified metabolic changes and physiological adaptations around calving, often associated with extensive mobilization of body reserves *post partum* in order to maintain energy balance (Bauman and Currie 1980; Bell 1995). During early lactation, mammals typically go through periods of negative energy balance (NEB) which are considered as normal for adapting to the homeorhetic changes after parturition.

However, cows that are selected for high milk production often experience a more profound and longer NEB compared to other mammalian species (Roberts and Coward 1984; Dewey 1997; Block et al. 2001).

Before parturition, nutrient requirements of the cow represent its own maintenance and that of the gravid uterus, consisting of the uterine tissue, placenta, fetal membranes and the conceptus. The "weigh-specific oxygen consumption" – representative for embryo(s) metabolic rate – is approximately twice that of the dam (Reynolds et al. 1986). The energy demands by fetuses and placenta peak during the last three weeks before parturition. Requirements for fetal growth are supplied mostly through glucose and amino acids since the placental transport of short- and long-chain fatty acids and ketones is limited in ruminants (Bell 1993); that is why maternal adaptations for carbohydrate, protein and lipid metabolism change during gestation. These changes are mainly characterized by increased hepatic gluconeogenesis accompanied by reduced glucose utilization in peripheral tissues (Bell 1995).

During late gestation, insulin responsiveness and insulin sensitivity of the glucose metabolism decrease, especially in overconditioned cows (de Koster et al. 2015). On a molecular basis, de novo lipogenesis and re-esterification of fatty acids are down regulated in adipocytes at the beginning of lactation, when immense lipolysis takes place (McNamara 1991; Vernon 2005). On the other hand, lipolytic pathways (basal and catecholamine-stimulated) are upregulated in insulin resistant adipocytes (Vernon 2005). Insulin acts as an antilipolytic hormone; given that low insulin levels are associated with the complete suppression of adipose lipogenesis (Kahn 1978). In order to encourage the mobilization of fatty acids from AT and to spare glucose utilization in peripheral tissues, a moderate degree of insulin resistance takes place in ruminants during late pregnancy and early lactation (Bell 1995). This insulin resistance during the transition period supports the increased demand in energy and nutrient flow to the mammary gland (de Koster et al. 2015). Consequently, glucose utilization by insulin sensitive organs like the AT and muscle is diminished, due to a decreased pancreatic insulin production (Petterson et al. 1994; Drackley et al. 2001).

In the transition from late pregnancy until lactation, the homeorhetic regulations also involve partitioning of nutrients with different tissue priorities (Bauman and Currie 1980). Milk synthesis after parturition goes along with an increased biosynthetic activity in the mammary gland and depends on the nutritional availability of substrates needed for producing the natural milk compounds which are predominantly lactose, protein and

triglycerides (Bell 1995). The demands for glucose, amino and fatty acids increase severalfold at the beginning of lactation compared with the requirements of the gravid uterus in late pregnancy. The fatty acids in milk triglycerides are either synthesized de novo in the mammary gland from acetate and ß-hydroxybutyrate (BHB) or taken up from plasma lipoprotein triglycerides (Bell 1995; Chilliard and Ferlay 2004). When nutrient intake cannot cover the energy requirements after parturition, considerable amounts of energy stores mainly from AT and skeletal muscle are mobilized in favor of producing milk for the offspring (Grummer et al. 2004). Within the first 8 weeks of lactation, cows mobilize on average 41.6 kg body reserves including 30.9 kg of body fat (Tamminga et al. 1997). The mobilization of long-chain fatty acids from AT is mediated by an increasing ratio of circulating growth hormone to insulin in order to compensate the deficit in energy intake (Pullen et al. 1989). High amounts of circulating fatty acids after parturition provide a considerable portion for the mammary glands milk fat synthesis (Pullen et al. 1989; Miller et al. 1991; Grummer 1993) and can also be used as additional energy source by other peripheral tissues (Herdt 2000).

In response to the diminished glucose availability due to the insufficient feed intake (FI) after parturition, subsequent lipolysis lead to an elevated release of NEFA and ketone bodies into the circulation (McNamara 1991; Herdt 2000). The NEFA entry rate into the bloodstream mainly represents the fat mobilization from the AT and in consequence the body fat loss, primarily in the first weeks after parturition (Dunshea et al. 1988; Bell 1995). The concentration of circulating NEFA and BHB can also be elevated during late pregnancy, even in animals carefully fed to predicted energy needs (Petterson et al. 1994). This is due to an decrease in the voluntary FI ( about 10-30% compared with FI during the early dry period in cows) that occurs normally around parturition in mammalians (Friggens 2003). Declining levels of progesterone, accompanied by increasing estrogen concentrations in this period may induce anorexia causing the decrease in FI before delivery (Muir et al. 1972; Grummer 1993).

The changes in circulating metabolites can be used to identify animals undergoing diseases in a subclinical state before exhibiting clinical symptoms (Huzzey et al. 2011). Parturition is associated with inflammatory processes; acute phase proteins such as haptoglobin have been described as possible biomarkers of inflammation around calving (Hachenberg et al. 2007). Haptoglobin is almost undetectable in clinical healthy animals, whereas high haptoglobin concentrations have been associated with an activated innate immune system

causing impaired reproductive efficiency in *post partum* dairy cows (Nightingale et al. 2015). Cows that are unable to adapt to a pronounced NEB are more susceptible to inflammatory processes and periparturient disorders (Duffield 2000; Drackley et al. 2005). In this context, the BCS before calving plays a crucial role in terms of the extent in mobilizing energy stores and an adequate nutrient intake after calving (Roche et al. 2009).

### *1.2 Body condition in dairy cows and its adaptation to the periparturient period*

The BCS defines the nutritional state in the cow (phenotypic characteristic) and is an important management tool in dairy farms (Schröder and Staufenbiel 2006). Further, the BCS at calving is an important determinant of early lactation milk yield and dry matter intake (Roche et al. 2009). The visual evaluation of the BCS is widely recognized as useful indicator for assessing energy reserves in cattle and can be easily implemented in big dairy herds. The BCS in German dairy herds is commonly categorized using a scale between 1 and 5 (with 0.25 intermediate steps; Edmonson et al. 1989), assessing cows from being severe underconditioned (emaciated; 1.0) to extreme overconditioned (obese; 5.0). The evaluation of the BCS is based on the external appearance of different defined locations of the cow accompanied by a tactile evaluation of the body (Edmonson et al. 1989; Schröder and Staufenbiel 2006). The characterization of body fat distribution via BCS is an easy tool and integrated in many farms, but however very subjective since the BCS is often underestimated in older, and obese cows (Schröder and Staufenbiel 2006).

Body fat can also be assessed through the measurement of the subcutaneous backfat thickness (BFT) of the sacral region by ultra-sonographic examination. Changes in the BFT reflect very well the shift between anabolism and catabolism during the periparturient period of dairy cows (Schröder and Staufenbiel 2006). Depending on the nutritional status, the BFT strongly correlates with the BCS in dairy cows ($r \geq 0.91 \leq 0.95$; Wittek and Fürll 2002), with one BCS unit being equivalent to about 10 mm BFT and about 50 kg total body fat (TBF; Schröder and Staufenbiel 2006).

Achieving homeostasis during the transition from late pregnancy to lactation represents a monumental task in modern high-producing dairy cows (McNamara 1991; Bell 1995). Hence, the management of BCS in dairy herds is crucial for maintaining healthy and productive cows. Feeding has to be adjusted to the individual needs of the cow to develop appropriate BCS before parturition in order to maintain the special needs during pregnancy

and lactation. Poor management during the dry period, but also environmental circumstances can lead to suboptimal body condition development in cows (Ingvartsen et al. 2003). On the other hand, individual FI, feed utilization, milk yield, but also genetic predisposition are significant factors influencing differences in the body condition of cows, even if they are subjected to the same management conditions (Rocco and McNamara 2013; Dechow et al. 2017).

Over- or under-conditioning before calving can negatively affect milk yield, immunity and fertility in cows and has been intensively researched over the past decades (Treacher et al. 1986; Agenäs et al. 2003; Hoedemaker et al. 2009; Akbar et al. 2015; Roche et al. 2015). Especially, during the dry period overconditioning or overfeeding is associated with severe BCS loss after calving, accompanied by a more pronounced and prolonged reduction of FI or NEB during early lactation, respectively, and an increased incidence of periparturient health disorders including hyperketonemia (Treacher et al. 1986; Agenäs et al. 2003; Roche et al. 2015; Mann et al. 2015) and oxidative stress (Bernabucci et al. 2005) compared to normal conditioned cows. An inadequate nutrient intake and the resulting NEB after calving causes elevated lipolysis in overconditioned cows, resulting in increased circulating concentrations of metabolites such as NEFA compared to normally conditioned cows (Agenäs et al. 2003; Hoedemaker et al. 2009).

Elevated circulating NEFA *post partum* (e.g., ≥ 0.7 mmol/L) are associated with a higher risk of developing periparturient health problems (e.g., displaced abomasum, clinical ketosis (CK), metritis) and culling (Ospina et al. 2010; McArt et al. 2013b). The rate limiting incomplete hepatic oxidation of NEFA induces the production of ketone bodies (e.g., acetone, acetoacetic acid) which are then released into the circulation (Herdt 2000). Ketone bodies and the oxidative product of acetoacetate, BHB, can also be used as substrate by other peripheral tissues (Bell 1981) or in response to the diminished glucose availability be taken up by the liver (Herdt 2000).

The limited ability of ruminants to export triacylglycerols (TAG) from the liver as very low density lipoproteins (VLDL) can lead to fat accumulation in the liver and hyperketonemia (Herdt 2000). The greater accumulation of TAG in the liver leads to hepatic lipidosis which is more frequently observed in overfed cows (Marcos et al. 1990; van den Top et al. 1996). In addition, hepatic lipidosis or fatty liver is associated with impaired fertility (Reid et al. 1983) and, as observed in overfed cows, also results in changes in the fatty acid profile in the liver (Rukkwamsuk et al. 1999) and circulating major fatty acids (e.g., palmitic, stearic, oleic,

and linoleic acids) compared with cows fed a restricted ration during the dry period (Rukkwamsuk et al. 2000). The severity of hyperketonemia has been characterized in several studies as either subclinical ketoses (SCK), when cows exceed a threshold of circulating BHB ≥ 1.2 mmol/L and not exhibiting visual signs; or CK, characterized by clinical signs such as reduced milk yield, inappetence, and weight loss (McArt et al. 2013b) with BHB concentrations exceeding ≥ 2.5 mmol/L (McArt et al. 2013a; Schulz et al. 2014; Mann et al. 2015). It has been reported that the peak prevalence of SCK occurs 5 weeks a.p., with SCK typically lasting about 5 days (McArt et al. 2012). In addition, cows that exceed the threshold of 0.96 mmol/L BHB in blood within the first 3 to 14 days *post partum* had an increased risk for diseases such as displaced abomasum, CK, metritis and retrained placenta (Ospina et al. 2010). However, in addition to an elevated BCS before calving (>3.5; 5-point scale), increased NEFA concentrations a.p. (≥ 0.30 mmol/L), calf sex, calving ease ≥ 3 (scale 1-5), stillbirths, parity ≥ 3, age, and season may be other significant factors influencing the risk of developing periparturient hyperketonemia (McArt et al. 2012).

In addition, cows with multiple parities (>3) exhibit greater body fat accumulation compared to cows of first or second parity (Schröder and Staufenbiel 2006; Roche et al. 2007). Consequently, primiparous cows show less lipolysis after parturition, resulting in reduced circulating NEFA and BHB, but higher insulin concentrations compared with multiparous cows (Gärtner et al. 2019). Moreover, obese multiparous cows tend to have greater mobilization of body reserves compared to normal-weight cows of the same parity (Gärtner et al. 2019).

The increased susceptibility to excessive mobilization of body fat reserves of overconditioned cows was attributed to elevated lipolytic activity with increasing adipocyte volume (de Koster et al. 2016). High adiposity in dairy cows before calving lowers the whole-body response to insulin, resulting in decreased expression of lipogenic genes in AT before parturition (Karis et al. 2020). Many decades ago, extreme obesity in cows was characterized as "fat cow syndrome", which was associated with an increased incidence of periparturient infectious disease and an extensive fatty liver syndrome (Morrow 1976). In contrast, human obesity and the accumulation of fat in different parts of the body has been linked to an increased risk of developing cardiovascular disease, type 2 diabetes, and certain cancers (Tchernof and Després 2013). In addition, increased release of thyroid hormones in obese human patients has been related to changes in body weight and metabolic rate compared with normal-weight patients (Reinehr 2010).

To investigate possible effects (e.g., emergence of periparturient diseases) resulting from extreme lipolysis after parturition associated with the increasing release of metabolites into the circulation, experimental approaches were applied to simulate the origin of these based on differential feeding before parturition. Cows calving at moderate BCS (3.0; 5-point scale) and fed *ad libitum* during the dry period increased their FI at a slower rate after parturition compared to cows fed restricted rations (Drackley et al. 2001). In addition, cows on restricted feeding had less liver TAG on d 1 *post partum* compared with cows on *ad libitum* feeding (Drackley et al. 2005). Dairy cows fed a high energy diet during the far-off period exhibited greater degrees of insulin resistance along with elevated blood NEFA concentrations during transition from late gestation to early lactation compared to cows fed a moderate energy ration (Holtenius et al. 2003; Drackley et al. 2005).

With the commencement of milk synthesis, the metabolic demands of high yielding dairy cows are tremendous. In the early weeks after calving, the AT plays a central role in whole-body energy metabolism and immunity by supporting adaptations to extreme metabolic changes during periods of nutritional limitations.

### *1.3 The adipose tissue*

The AT is a loose connective tissue composed of fat cells (adipocytes) capable of storing energy as triglycerides during times of nutrient oversupply. Consequently, these energy stores can be used in periods of nutritional deficiency to increase survivability in higher organisms, including mammals. Extreme energy storage in AT is prevented within the physiological range of energy balance regulation by reducing feed intake and increasing energy expenditure to avoid obesity. This implies a feedback system involving hormones and proteins that mediate the amount of energy stored in the AT (Spiegelman and Flier 1996). In addition to its central role of storing and releasing energy (Figure 1), the AT expresses and secretes various hormones, including steroids (Siiteri 1987) and numerous other bioactive peptides, that are referred to as adipokines and act at endocrine, autocrine and paracrine levels (Kershaw and Flier 2004). Moreover, the AT can influence the metabolism of other organs, including the liver, through the secretion of proteins and hormones (Chilliard et al. 2005; Vernon 2005).

Leptin, adiponectin, and chemerin are known signaling proteins synthesized and secreted by the AT, exerting key functions in regulating steroidogenesis in the gonads (Campos et al. 2008). The proteohormone leptin was first described more than 20 years ago in rodents as

a product of the *ob* gene (Zhang et al. 1994). Leptin is produced mainly by adipocytes, whereas circulating leptin is proportional to the amount of stored lipids in the white AT (Considine et al. 1996). The hormonal action of leptin is to regulate appetite and stimulate energy expenditure (Ahima et al. 1996; Friedman and Halaas 1998). Leptin also stimulates other metabolic hormones that act on peripheral tissues by increasing insulin sensitivity, glucose utilization, and fatty acid oxidation in tissues (Chilliard et al. 2005). In dairy cows, circulating leptin is positively associated with body fat content, energy balance, and feeding intensity (Chilliard et al. 2001; Kokkonen et al. 2005).

After parturition, diminished FI is accompanied with significantly reduced leptin secretion and increasing NEFA levels (Accorsi et al. 2005). A fast reduction in circulating leptin – as a result of malnutrition – has been attributed to decreased thyroid activity and energy expenditure in animals, which stimulates glucocorticoid (GC) secretion (Friedman and Halaas 1998; Chilliard et al. 2001; Block et al. 2001).

As lactation proceeds, leptin rises again alongside increasing energy balance and serves as a "metabolic signal" for the level of nutritional status (Accorsi et al. 2005). The "insulin-sensitizing" effect of leptin, leading to enhanced fatty acid oxidation, may be the main reason for the stimulation of lipolysis in AT (Chilliard et al. 2005). On the other hand, it has been reported that leptin secretion by adipocytes is both stimulated (Brann et al. 1999) and inhibited (Abelenda and Puerta 2004) by sex steroids such as estradiol and progesterone. During the last trimester of human pregnancy, the attenuated increase in circulating leptin is due to increasing progesterone concentrations, which have an inhibitory effect on leptin secretion by adipocytes (Schubring et al. 1998; Hardie et al. 1997). This could be part of an adaptation during pregnancy in order to accumulate fat depots (Abelenda and Puerta 2004) for times of high energy demand after parturition.

Adiponectin, expressed by hepatocytes and adipocytes, is the most abundant adipokine and is involved in glucose and lipid metabolism due to its insulin-sensitizing effect (Tschritter et al. 2003; Yamauchi et al. 2002). The potential effector regulating adiponectin concentration in dairy cows has been attributed to energy balance around parturition (Krumm et al. 2017).

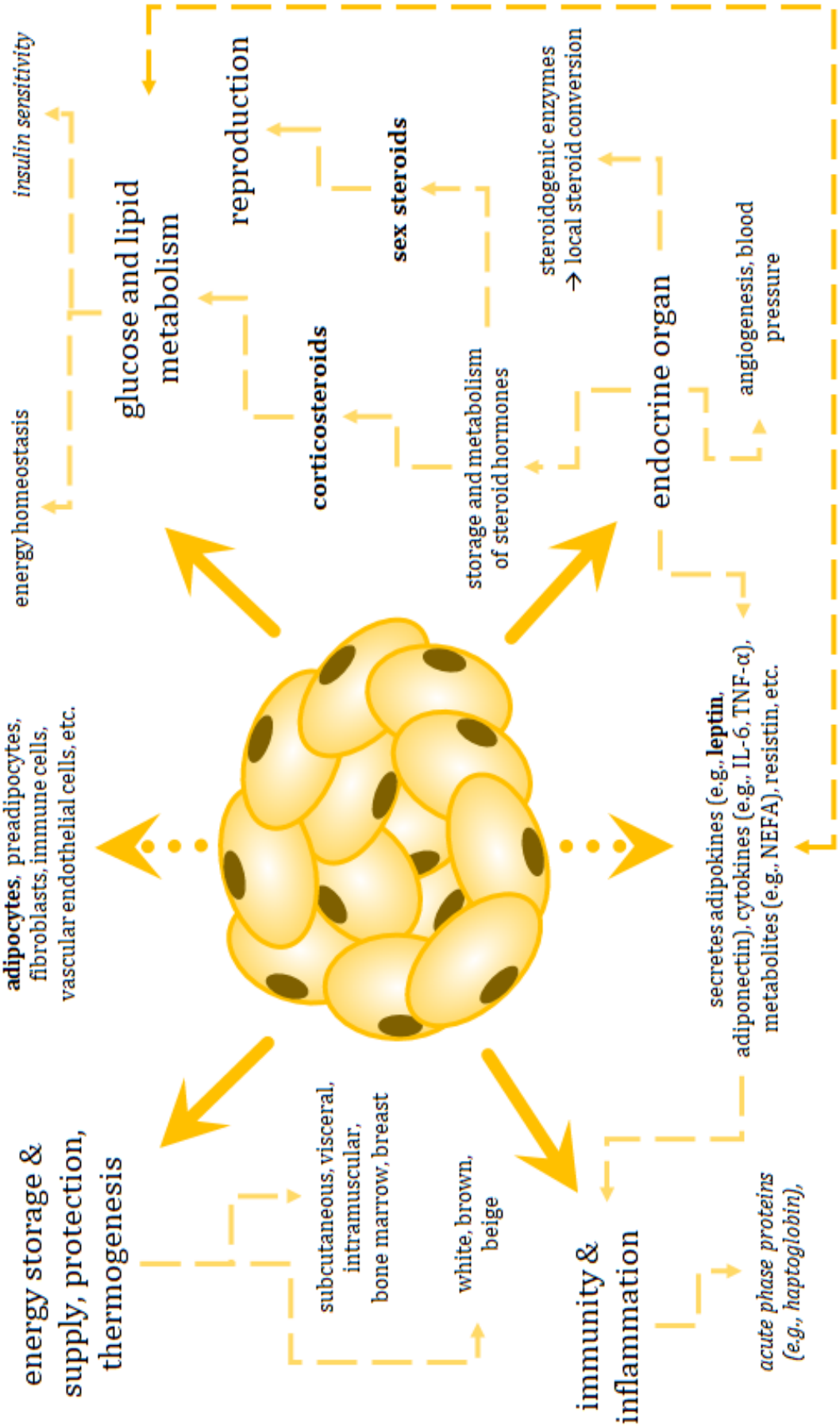


**Figure 1** Schematic presentation of adipose tissue composition and action. Abbreviations: IL-6, Interleukin-6; TNF-α, tumor necrosis factor alpha; NEFA, non-esterified fatty acids. Self-designed illustration (modified from Ahima and Flier 2000; Luo and Liu 2016).

In gravid dairy cows, adiponectin decreases toward calving, with concentrations reaching a nadir during the first weeks of lactation (Singh et al. 2014; Sauerwein and Häußler 2016). Decreasing adiponectin concentrations reflect the adiponectin concentration in AT, which contributes to increasing lipolysis (Singh et al. 2014). Furthermore, in lactating dairy cows, the adiponectin protein was detected at higher levels in visceral versus subcutaneous AT depots, suggesting that AT depots also contribute differentially to adiponectin in circulation (Singh et al. 2014).

In addition to the secretion of adipokines, AT also contributes to the whole-body steroid levels and local conversion of steroids through the action of steroidogenic enzymes (Campos et al. 2008; Kershaw and Flier 2004).

### *1.4 Steroid hormones*

Steroid hormones are lipophilic molecules produced mainly in the adrenal cortex, gonads, or placenta and can be classified as either corticosteroids or sex steroids. Underlying, steroids are further classified into 5 types according to their receptor binding affinity: progestagens (gestagen), mineralo- and glucocorticoids (corticosteroids), androgens and estrogens (see figure 2). Steroid hormones differ in the groups attached to the ring system and in changes to the ring itself: e.g., double bonds, an aromatic ring instead of the aliphatic one. Cholesterol is the most important sterol of animal, plant, and bacterial organisms and contains sterane, consisting of 4 carbon rings, as its basic skeleton.

Cholesterol is formed from squalene on the basis of mevalonic acid. The last intermediate from which cholesterol is finally formed is 7-dehydrocholesterol, which can also be converted to vitamin $D_3$ (Träger 1977). Steroid hormones are synthesized *de novo* from cholesterol in classical steroidogenic tissues (such as adrenals, gonads, and placenta; Payne and Hales 2004). For this, cholesterol is initially transferred from the cytosol to the inner mitochondrial membrane, where the steroidogenic acute regulatory protein (**StAR**) and CYP11A1 (cytochrome P450 cholesterol side-chain cleavage family 11 subfamily A member 1; also referred as P450scc) catalyzes the cleavage of the cholesterol side chain to form pregnenolone (Payne and Hales 2004; Figure 2). In this process, cholesterol transfer in mitochondria is also supported by other mitochondrial proteins, including the translocator protein (TSPO; Hauet et al. 2005).

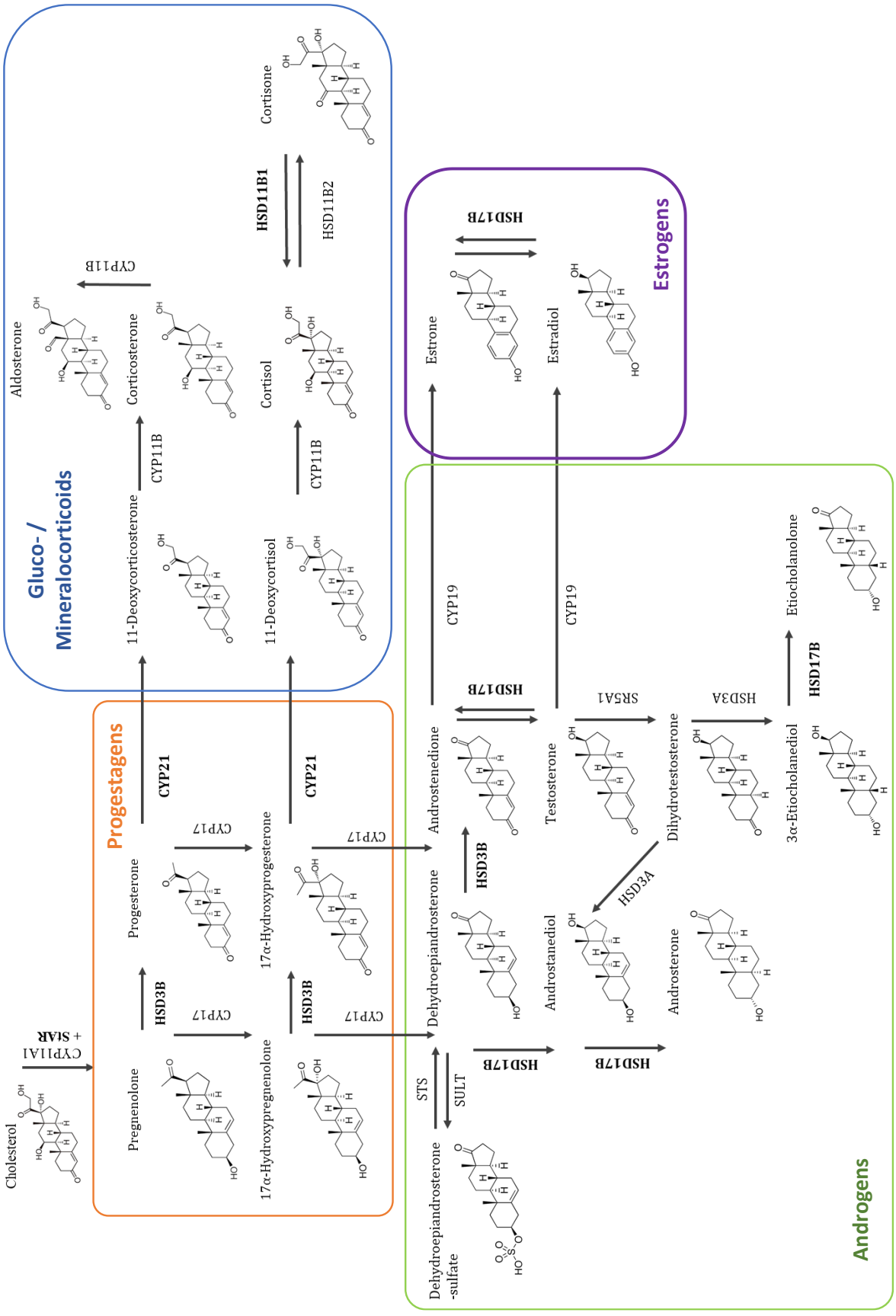


**Figure 2:** Biosynthetic pathway for progestagens, gluco- and mineralocorticoids, androgens, and estrogens. Enzymes involved in the steroidogenic pathway are shown next to the arrows, those enzymes being detected in the present study were highlighted in bold (the Figure was adapted from Figure 1 in MacKenzie *et al.* (2008). CYP11A1: cholesterol monooxygenase, CYP11B2: aldosterone synthase, CYP17: steroid-17α-hydroxylase, CYP19: aromatase, CYP21: steroid 21-hydroxylase, HSD3A: 3α- hydroxysteroid dehydrogenase, HSD3B: 3 β-hydroxysteroid dehydrogenase, HSD11B1: 11 ß-hydroxysteroid dehydrogenase type 1, HSD11B2: 11 ß-hydroxysteroid dehydrogenase type 2, HSD17B12: 17 ß-hydroxysteroid dehydrogenase type 12, SR5A1: steroid-5α-reductase, StAR: steroidogenic acute regulatory protein, STS: steroid sulfatase, SULT: sulfotransferase.

The main biological functions of steroid hormones are: [1] enhancement or maintenance of cellular functions in target organs and increase in protein synthesis and cell division (progestins, estrogens, androgens, corticosteroids), [2] reduction of certain cellular functions associated with a decrease in cell growth or cell number in a tissue (catabolic action of glucocorticoids), [3] negative or positive feedback on the secretion of liberins and tropines (all steroid classes), [4] direct interactions of steroids with membranes (part of the anti-inflammatory effect of corticoids and membrane-stabilizing effect of androgens and estrogens), [5] central effect on animal behavior influenced by extra-hypothalamic brain regions (androgens, estrogens, progestogens, and possibly corticoids), [6] organizational effect in fetal development and differentiation of sex-specific enzyme patterns in various organs (androgens, corticoids). The regulated transcription of steroid hormone action is mediated by binding to their specific receptors, inducing biological responses (Träger 1977).

In addition to the classical steroid hormones (corticosteroids and sex steroids), there are several subclasses of steroids such as oxysterols (oxy-derivatives of sterols or steroids) and secosteroids, which also include vitamin D (Li et al. 2015). In general, ovaries and testes are the main source of estrogens and androgens, respectively, in mammalian species.

However, the liver is one of the main organs responsible for steroid metabolism (Payne and Hales 2004) while also the AT is known to be an important reservoir for the lipophilic steroid hormones, which are locally metabolized by steroidogenic enzymes (Bélanger et al. 2002; Deslypere et al. 1985). Locally produced hormones exert their action within the same cell (intracrine), which is different from the general paracrine, autocrine and endocrine systems (Labrie 1991, 1993). The transport of steroid hormones, which are considered to act in an endocrine manner, is mediated by sex hormone-binding globulins (Lin et al. 2010). In contrast to the endocrine functions, intracrine activity requires minimal amounts of a hormone to achieve maximal output (Labrie 1991). Consequently, the local requirement for steroid hormones (Blouin et al. 2009; Bélanger et al. 2002) and oxysterols (Li et al. 2015) in peripheral tissues, including the AT, is generated depending on the presence of specific steroidogenic enzymes (Labrie 1991; Labrie et al. 1997).

### 1.4.1 Steroidogenesis and steroidogenic enzymes

The first step of steroidogenesis is rate-limited by the action of the steroidogenic enzyme CYP11A1. When CYP11A1 is not expressed in a tissue, *de novo* steroidogenesis is abolished (Miller and Bose 2011). As described above, CYP11A1 catalyzes the cleavage of the cholesterol side chain to generate pregnenolone from cholesterol (Payne and Hales 2004). The hydroxysteroid dehydrogenase (HSD) family is primarily responsible for the oxidation and reduction of steroid hormones. While enzyme activity differs between tissues, HSD-enzymes are essential for the transformation of inactive to active steroids (Chen et al. 2012).

The superfamily of short-chain alcohol dehydrogenase reductases includes both 3ß-hydroxysteroid-dehydrogenase (**HSD3B**) and 17ß-Hydroxysteroid dehydrogenase (**HSD17B**) enzymes (Payne and Hales 2004). Pregnenolone is converted to progesterone mainly by the mitochondrial form of HSD3B (MacKenzie et al. 2008). Not being synthesized *de novo*, pregnenolone can also exit the mitochondria and be further metabolized to 17a-hydroxyprgnenolone by 17α-hydroxylase (**CYP17**) in the endoplasmic reticulum (ER; Li et al. 2015).The CYP17 enzyme also catalyzes the synthesis of DHEA and androstenedione from pregnenolone and progesterone, respectively (Figure 2). The presence of CYP17 enables a cell to produce sex steroids (Li et al. 2015). CYP17 mRNA has been shown to be present in the abdominal scAT of premenopausal woman (Puche et al. 2002).

Progesterone is the most important functional gestagen produced and secreted by the corpus luteum and placenta during the estrous cycle and pregnancy. Progesterone supports pregnancy and embryogenesis in mammals and is required for the development of mammary tissue (mammogenesis) for the commencement of milk production. Milk and systemic blood levels of progesterone are highly correlated ($r > 0.8$) (Abeyawardene et al. 1984). Therefore, the changing concentration of progesterone in the milk of dairy cows has been used for decades to monitor luteal function (Heap et al. 1973). From pregnenolone onwards, the classical steroidogenic pathway can branch in different directions to yield either mineralo- and glucocorticoids or sex steroids under the action of HSD3B and steroid 21-hydroxylase (CYP21; Li et al. 2015).

The enzyme HSD3B is essential for the formation of all active steroid pathways and also converts the Δ5-steroid precursors 17α-hydroxypregnenolone, DHEA, and androstenediol into their respective Δ4-ketosteroids 17-OHP, androstenedione, and testosterone (Li et al. 2015). Both isoforms of HSD3B (type 1 and 2) mRNA have been detected in human AT (Blouin et al. 2008; MacKenzie et al. 2008). Androstenedione, DHEA, and DHEA-S are referred to as precursor steroids since they are not biologically active hormones and have to be converted into active androgens or estrogens (e.g., dihydrotestosterone (DHT) or estradiol (E2), respectively (Figure 2) by the action of steroidogenic enzymes such as 3HSDB, 17HSDB, 5α-reductase, or aromatase (Labrie 1991). As steroid precursors, DHEA-S and estrone-sulfate are hydrolyzed locally by the enzyme steroid sulfatase (STS) to DHEA and estrone, respectively (Reed et al. 2005). Sulfonated steroids such as DHEA-S, pregnenolone-sulfate, and estrone-sulfate play a central role in terms of an inactive reservoir for unconjugated steroids in human steroidogenesis as they reach high concentrations in circulation (Labrie 1991; Neunzig et al. 2014). However, in contrast to DHEA, circulating DHEA-S in the blood of dairy cows shows only low levels during mid lactation (Marinelli et al. 2007).

Also destined for renal clearance, sulfonated steroids may be an end product of xenobiotic metabolism (Scrott 1996). In dairy cows, estrone-sulfate is formed mainly in the conceptus with increasing concentrations during gestation (Hoffmann et al. 1997), while estrone-sulfate in urine can be used to monitor pregnancy (Yang et al. 2003).

The conversion of androgens to estrogens is initiated by aromatase activity (product of the CYP19A1 gene) and has been detected (mRNA expression) in various human tissues including visceral and subcutaneous AT (Stocco 2012). The mRNA expression of CYP19A1 in adipose stromal cells is regulated by cytokines (e.g., interleukin 6 and tumor necrosis factor alpha) and glucocorticoids (Simpson 2004) as well as leptin (Brown et al. 2009). Thereby, the expression of CYP19 is dependent on the origin of the fat depot (scAT higher expression than visceral in obese men and premenopausal women; Wang et al. 2013; Li et al. 2015). Estrogens can stimulate the development of mammary ducts and proliferation of secretory tissue (Erb 1977). Estrogens, mainly produced in the placenta during gestation, are essential for maintaining pregnancy (Wendorf et al. 1983; Thorburn and Challis 1979) and for inducing parturition in dairy cattle (Hoffman et al. 1979).

The irreversible reduction of testosterone to the most potent androgen dihydrotestosterone (DHT) is catalyzed by 5α-reductases *(types 1 and 2: SRD5A1 and SRD5A2)*. While SRD5A2 is predominately found in male reproductive tissues, SDR5A1 is also expressed in peripheral tissues including the AT (Russell and Wilson 1994; Li et al. 2015).

The final steps in the formation of androgens and estrogens are controlled by isoforms of the enzyme HSD17B. To date, 14 isoforms of the HSD17B family have been identified in vertebrates and belong to either the superfamily of short-chain dehydrogenase/reductases (SDRs) or the aldoketo reductases (AKRs; Type 5), which activate or inactivate estrogens or androgens, respectively (Moeller and Adamski 2009). The major role of HSD17 enzymes in either activating or inactivating potent sex steroids makes them crucial for reproductive tissue function, growth, and development in both sexes (Labrie et al. 1997). Given the diversity of the different isoforms and their broad tissue distribution (Table 1), the HSD17 enzymes deserve more intensive consideration in this thesis.

In addition to their steroidogenic activity, the various HSD17 isozymes partially have broad substrate affinities and cell expression, catalyic preferences, and different subcellular localisation that characterize HSD17 enzyme activities (Adamski and Jakob 2001; Moeller and Adamski 2009). Different types of HSD17B have been described as an reductive (estrogenic) substance catalyzing the conversion of estrone (E1) to estradiol (E2; Table 1). The estrogenic activity of HSD17B was investigated in 25 different human tissues, with the highest enzyme activity found in placenta, liver, ovary, endometrium, prostate, testis, and adipose tissue. The latter tissues also likely have favorable estrogenic pathways that prioritize the formation of E2 and adjust the rate of steroids formed to local needs (Labrie et al. 1997).

The present work focuses on isotype 12 of HSD17B (**HSD17B12**), since it has been described to be involved mainly in the conversion of E1 to E2 and in the elongation process of branched- and long chain-amino acids (Moon and Horton 2003; Luu-The et al. 2006; Blanchard and Luu-The 2007; Moeller and Adamski 2009) making it the most favorable isotype to reach the aim of this work. In addition, HSD17B12 has been detected in human (Bellemare et al. 2009) and murine fat cells (Blanchard and Luu-The 2007) as well as in a variety of other tissues (see table 1). Nevertheless, it is still unclear whether HSD17B12 is more involved in sex steroids conversion or lipid metabolism (Moeller and Adamski 2009).

The conversion from progestins to mineralo- and glucocorticoids is triggered by the enzyme steroid 21-hydroxylase (CYP21; Figure 2). Microsomal CYP21 initiates 21-hydroxylation of progesterone or 17α-hydroxyprogesterone to deoxycorticosterone or 11-deoxycortisol, respectively (Miller and Auchus 2011). Gene expression, but not the enzyme activity of CYP21 was detected in human visceral and subcutaneous fat cells (MacKenzie et al. 2008). The enzyme 11ß-hydroxysteroid dehydrogenase type 1 (**HSD11B1**) acts mainly as a reductase, producing the biologically active cortisol from the inactive cortisone. The type 2 of HSD11B works in the opposite way, inactivating cortisol. The enzyme is highly expressed in human visceral fat (Stulnig and Waldhäusl 2004) and has also been detected in various AT depots in dairy cows (Friedauer et al. 2015).

Many aspects of glucose homeostasis are mediated by GC; e.g., they promote gluconeogenesis in the liver (Kraus-Friedmann 1984) or decrease glucose uptake and utilization in muscle and AT by antagonizing the insulin response (Kuo et al. 2013). The GC have also been reported to be involved in inflammatory responses (Coutinho and Chapman 2011) and to contribute to the differentiation of pre- to major adipocytes (Tomlinson et al. 2004). Increased GC in the circulation lead to hyperglycemia and cause insulin resistance (Di Dalmazi et al. 2012). Nevertheless, GC preserve glucose for maintaining maximal brain function during times of stress (e.g., starving or fasting) since glucose serves as the brain´s main source of energy (Charmandari et al. 2005).

The final products of the mineralo- and glucocorticoid pathway are formed with the mitochondrial enzymes 11ß-hydroxylase (CYP11B1) and aldosterone synthase (CYP11B2). The latter enzyme exclusively catalyzes the conversion of aldosterone, whereas CYP11B1 triggers the transformation of 11-deoxycortisol and deoxycorticosterone to cortisol and corticosterone, respectively (Payne and Hales 2004). Aldosterone, CYP11B1, and CYP11B2 (mRNA and protein) were found in mature human and murine adipocytes (Briones et al. 2012).

**Table 1:** Specifics and tissue distribution of human 17ß-hydroxysteroid dehydrogenase isoforms

| HSD17 -type | Gene name | Substrate specifity [a, †] | Oxidative / reductive [d] | Tissue distribution [e, †] | Subcellular localization [†] |
|---|---|---|---|---|---|
| 1 | HSD17B1 | Estrogens, androgens | Reductive | Placenta, ovary, endometrium, breast | Cytosol |
| 2 | HSD17B2 | Estrogens, androgens, progestins | Oxidative | Liver, intestine, endometrium, placeta, pancreas, prostate, colon | Endoplasmatic reticulum (ER) |
| 3 | HSD17B3 | Androgens, estrogens | Reductive | Testis (brain, blood, skin, **adipose tissue**) | ER |
| 4 | HSD17B4 | Very long chain fatty acids, branched fatty acids, bile acids, estrogens[b], androgens[b] | Oxidative | Liver, heart, prostate, testis, lung, skeletal mucle, kidney, pancreas, thymus, ovary, intestine, placenta, brain, spleen, colon, lymphocytes | Peroxisomes |
| 5 | AKR1C3 | Androgens, progestins, estrogens, prostaglandin | Both | Prostate, mammary gland, liver, kidney lung, heart small intestine, colon, uterus, testis, brain, skeletal muscle, **adipose tissue** | Cytosol |
| 6 | HSD17B6 | Androgens, estrogens[c] | Both | Liver, testis, lung, spleen, brain, ovary, kidney, adrenal, prostate | Microsomal |
| 7 | HSD17B7 | Sterols, estrogens, androgens, progestins | Reductive | Ovary, uterus, placenta, liver, breast, testis, neuronal tissue, adrenal gland, small intestine, lung, thymus, prostate, **adipose tissue** | ER |
| 8 | HSD17B8 | Estrogens, androgens | Oxidative | Prostate, placenta, kidney, brain, cerebellum, heart, lung, small intestine, ovary, testis, adrenal, stomach | Mitochondria |
| 9 | HSD17B9 | Retinol dehydrogenase | Oxidative[∞] | *unknown* | *unknown* |
| 10 | HSD17B10 | Short chain fatty acids, branched chain fatty acids, bile acids, estrogens, androgens, progestins, corticosteroids | Oxidative | Liver, small intestine, colon, kidney, heart, brain, placenta, lung, ovary, testis, spleen, thymus, prostate, peripheral blood leukocyte | Mitochondria |
| 11 | HSD17B11 | Estrogens, androgens | Oxidative | Liver, pancreas, intestine, kidney, adrenal gland, heart, lung testis ovary, placenta, sebaceous gland | ER, lipid droplets |
| 12 | HSD17B12 | Branched and long chain fatty anids, estrogens, androgens[c] | Reductive | heart, skeletal muscle, liver, kidney, adrenal gland, testis, placenta, cerebrum, pancreas, stomach, small intestine, large intestine, trachea, lung and thyroid, esophagus, prostate, aorta, urinary bladder, spleen, skin, brain, ovary, breast uterus, vagina, **adipose tissue[#]** | ER |
| 13 | HSD17B13 | Unknown | unknown | Liver (bone marrow, lung, ovary, testis, kidney, skeletal muscle brain, bladder) | ER, lipid droplets |
| 14 | HSD17B14 | Estrogens androgens | Oxidative[∞] | Brain, liver, placenta, breast | Cytosol |

Modified from Moeller and Adamski, (2009); [a] in human; [b] observed in pig; [c] observed in rodents; [d] cofactor preference either NADP/NADPH (reductive) or NAD/NADH (oxidative) or both; [e] by Nothern, RT-PCR or immunological methods; [∞] Lukacik et al. 2007; [#] Bellemare et al. 2009; [†] *original references of experimental data from substrate specifity and tissue distribution of HSD17 isoforms can be found in Moeller and Adamski (2009).*

### 1.4.2 Steroid metabolism in adipose tissue

Significant portions of steroid biosynthesis and metabolism occur in AT, primarily through the uptake of circulating precursor steroids. Sex steroids, including estrogens, for example, have high metabolic importance in the regulation of lipolysis and AT deposition at the local level (Cooke and Naaz 2004). Circulating steroid precursors (DEHA, DHEA-S, 4-dione, and testosterone) secreted by the adrenal gland or gonads can be taken up by the AT and are further metabolized to biologically active sex steroids by the action of steroidogenic enzymes (Bélanger et al. 2002). The synthesis and secretion of adipokines (e.g., leptin, adiponectin, and chemerin) into the circulation regulates adrenal and gonadal steroidogenesis (Kershaw and Flier 2004; Campos et al. 2008). Steroids are lipophilic hormones with a higher solubility in lipids than in aqueous media, which makes the importance of the relationship between the AT and steroids very clear.

Several decades ago, McCracken (1964) showed that progesterone concentrations in body fat of dairy cows are about 10-fold higher compared with plasma concentrations and may serve as a reservoir for maintaining normal progesterone concentrations in the bloodstream. During pregnancy, progesterone appears to accumulate until a level of saturation is reached within the AT (Hamudikuwanda et al. 1996). In humans, it has also been shown that various steroids are accumulated and stored in the AT in concentrations up to 400-fold higher than in the bloodstream (Deslypere et al. 1985). Therefore, measurement of local steroid concentration appears to be a more accurate indicator of steroid action within the tissue (Li et al. 2015).

The concentration of steroids in AT is determined by local formation through steroidogenic enzymes, which may lead to imbalances in terms of defects in steroidogenesis; that is, a shift in steroid concentration in peripheral tissues potentially leads to atypical production of adipokines, resulting in the pathogenesis of metabolic disorders (Tchernof et al. 2015). Moreover, AT metabolism is regulated by locally produced steroids and could also contribute quantitatively to holistic steroid levels in the body (Li et al. 2015).

To date, the influence of steroid hormones on adipose tissue function is not fully understood in human research (Blouin et al. 2009), and consequently even less so in terms of bovine adipose physiology.

### 1.4.3 The impact of body condition on steroidogenesis

For the maintenance of biological functions, e.g. the differentiation and proliferation processes of cells and tissues, proper functioning of whole-body steroid metabolism and biosynthesis is required (Chen et al. 2012). Defects in steroidogenesis and local formation of sex steroids have been linked to the development of human malignancies such as neurological diseases, cancer, or type 2 diabetes (Labrie 1993; Labrie et al. 1997).

Steroids are involved in modulating body fat distribution at the local level (Tchernof and Després 2013), with estrogens stimulating lipolysis by inhibiting lipoprotein lipase activity (Cooke and Naaz 2004) and androgens inhibiting fat cell differentiation (Blouin et al. 2009). It was also shown that genetically obese mice (db/db) secreted higher amounts of aldosterone compared with control mice (db/+), indicating obesity-induced upregulation of adipocyte-derived aldosterone that may cause hyperaldosteronism (Briones et al. 2012).

The shift from carbohydrate- to fat-based metabolism is regulated by increased circulating GC and is accompanied by a reduction in insulin as a consequence of reduced glucose levels, stimulating lipolysis (Ahima and Flier 2000). In addition, steroidogenic enzymes have been suggested to play an important role in obesity and fat accumulation (Bélanger et al. 2002), although the exact mechanisms of action are not yet known. Steroid conversion in human AT accounts for approximately one-third of peripheral androgen synthesis, with increasing importance with respect to obesity (Boulton et al. 1992). In murine adipocytes, the expression of steroidogenic enzymes (e.g., StAR) was increased during adipogenesis (Li et al. 2014).

The enzyme HSD11B1, also present in bovine scAT (Friedauer et al. 2015), has previously been related to obesity and insulin resistance in several species (Bujalska et al. 2002; Paulmyer-Lacroix et al. 2002; Draper and Stewart 2005). In addition, HSD11B1 mRNA expression in dairy cows was shown to correlate strongly with body weight and BCS at times of fat accumulation (Sultana 2015). Moreover, overexpression of HSD11B1 mRNA was positively correlated with the extent of obesity in humans (Paulmyer-Lacroix et al. 2002; Lee et al. 2014) or in genetically modified obese rats (Prasad et al. 2010). The potential influence of increased expression and activity of steroidogenic enzymes along with obesity has been associated with reproductive disorders in humans (Diamanti-Kandarakis 2007).

The importance of local uptake and conversion of steroid hormones in AT has been known for decades with regard to human medical research (Bélanger et al. 2002). In dairy cows, little is known about the release of steroids from AT and the expression of steroidogenic enzymes in response to changes in body condition. Nevertheless, the release of progesterone from the AT of dairy cows has been demonstrated by restricted feeding that stimulated lipolysis (Rodrigues et al. 2011; Ferraretto et al. 2014) and by the ß-adrenergic stimulation of adipose tissue explants *in vitro* (Hamudikuwanda et al. 1996).

Elevated progesterone levels along with an increase in circulating NEFA in dairy cows, were attributed to an increased loss of body condition and were associated with lower estrus activity *post partum* (Lüttgenau et al. 2016). On the other hand, changes in circulating progestins could also be due to local conversion by steroidogenic enzymes such as steroid 17α-hydroxylase (CYP17; Puche et al. 2002).

In this context, we wanted to investigate whether the release of steroid hormones from adipose tissue is affected by changes in body condition, which in turn may lead to an altered steroid profile when comparing normal and over-conditioned dairy cows.

## 2 Objectives and hypothesis

The effects of overconditioning before calving, followed by intense lipolysis after calving, have been the subject of extensive research in dairy cows over the past decades. The augmented release of metabolites and hormones as a result of intense lipolysis after calving may also lead to an increased release of steroids from the AT. So far, only certain parameters of the complex network of steroid metabolism in dairy cows have been selectively highlighted or the analysis has been limited to specific segments.

To the best of our knowledge, local steroid metabolism in AT (including expression of steroidogenic enzymes) in cows with different degrees of *post partum* tissue mobilization and comparison of serum and adipose steroid concentrations have not been studied previously. To gain insight into possible effects of varying degrees of *post partum* lipolysis on steroid metabolism (blood and scAT) in dairy cows, the objectives of this thesis were:

- to investigate the extent of lipid mobilization in cows with high (HBCS) versus normal body condition (NBCS) before calving by means of circulating metabolites (NEFA, BHB) after calving and to compare longitudinal changes in performance (milk-yield) as well as metabolic and endocrine parameters,
- to compare changes in serum and scAT steroid concentrations during the transition from late pregnancy to mid-lactation in HBCS versus NBCS cows, and
- to assess the mRNA expression of selected steroidogenic enzymes in liver and scAT and compare these expressions between HBCS and NBCS cows during the periparturient period.

It was hypothesized that HBCS cows would mobilize more lipid reserves than NBCS cows in addition to increased concentrations of circulating metabolites (e.g., NEFA, BHB) and hormones (e.g., leptin, thyroid hormones). We also expected that HBCS cows experience more oxidative stress than NBCS cows during early lactation. Further, it has been suggested that overconditioning prior to calving results in higher levels of circulating steroid hormones that are subjected to increased *post partum* AT mobilization compared to normal conditioned cows. In addition, mRNA expression of steroidogenic enzymes (liver and scAT) was thought to be related to the progression of circulating and stored steroids.

## 3 Manuscript I

(Published in **Animal** – The International Journal of Animal Biosciences, 2019, 13(7): 1478-1488, DOI: 10.1017/S1751731118003385)

# Comparison of performance and metabolism from late pregnancy to early lactation in dairy cows with elevated v. normal body condition at dry-off

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**Abstract**

Excessive mobilization of body reserves during the transition from pregnancy to lactation imposes a risk for metabolic diseases on dairy cows. We aimed to establish an experimental model for high v. normal mobilization and herein characterized performance, metabolic and endocrine changes from 7 weeks antepartum (a.p.) to 12 weeks *post partum* (p.p.). Fifteen weeks a.p., 38 pregnant multiparous Holstein cows were allocated to two groups that were fed differently to reach either high or normal body condition scores (HBCS: 7.2 NEL MJ/kg dry matter (DM); NBCS: 6.8 NEL MJ/kg DM) at dry-off. Allocation was also based on differences in body condition score (BCS) in the previous and the ongoing lactation that was further promoted by feeding to reach the targeted BCS and back fat thickness (BFT) at dry-off (HBCS: >3.75 and >1.4 cm; NBCS: <3.5 and <1.2 cm). Thereafter, both groups were fed identical diets. Blood samples were drawn weekly from 7 weeks a.p. to 12 weeks p.p. to assess the serum concentrations of metabolites and hormones. The HBCS cows had greater BCS, BFT and BW than the NBCS cows throughout the study and lost more than twice as much BFT during the first 7 weeks p.p. compared with NCBS. Milk yield and composition were not different between groups, except that lactose concentrations were greater in NBSC than in HBCS. Feed intake was also greater in NBCS, and NBCS also reached a positive energy balance earlier than HBCS. The greater reduction in body mass in HBCS was accompanied by greater concentrations of non-esterified fatty acids, and β-hydroxybutyrate in serum after calving than in NBCS, indicating increased lipomobilization and ketogenesis. The mean concentrations of insulin across all time-points were greater in HBCS than in NBCS. In both groups, insulin and IGF-1 concentrations were lower p.p than in a.p. Greater free thyroxine (fT4) concentrations and a lower free 3-3'-5-triiodothyronine (fT3)/fT4 ratio were observed in HBCS than in NBCS a.p., whereas p.p. fT3/fT4 ratio followed a reverse pattern. The variables indicative for oxidative status had characteristic time courses; group differences were limited to greater plasma ferric reducing ability values in NBSC. The results demonstrate that the combination of pre-selection according to BCS and differential feeding before dry-off to promote the difference was successful in obtaining cows that differ in the intensity of mobilizing body reserves. The HBCS cows were metabolically challenged due to intense mobilization of body fat, associated with reduced early lactation dry matter intake and compromised antioxidative capacity.

*Keywords:* bovine, pre-selection, dry period, body reserve, mobilization

## Implications

An experimental model for studying dairy cows that differ in the extent of peripartal mobilization of body reserves was successfully established. The model's key elements comprise preselecting cows for normal v. high body condition by 8 weeks before dry-off, and differential feeding of the two groups until dry-off to further increase or to maintain the body condition score (BCS). The targeted difference in mobilization of body reserves was sustained during the dry period and the subsequent 12 weeks of lactation. Concordant differences in blood metabolites and in two out of six metabolic hormones investigated were observed.

## Introduction

Overconditioned cows loose relatively more of their body condition in early lactation and have reduced DM intake (DMI) and, due to increased lipolysis, greater circulating concentrations of non-esterified fatty acids (NEFA) than thinner cows (Drackley et al., 2001). The NEFA and ketone bodies produced therefrom can be oxidized in several peripheral tissues in the body for generating energy and also serve as substrate for mammary fatty acid synthesis. When the liver's capacity for oxidation and export of NEFA is exceeded, NEFA are re-esterified to triglycerides and can thus lead to a fatty liver syndrome, while hyperketonaemia may result in ketosis (Drackley et al., 2001). Precalving body condition score (BCS) and precalving feeding level have been demonstrated to exert both interdependent and independent effects on production and health characteristics of transition dairy cows (Roche et al., 2015). We are particularly interested in studying cows that differ in the extent of mobilizing body reserves and thus our main objective was to elaborate an animal model to obtain cows differing in BCS already at dry-off.

For achieving this goal, we pre-selected cows based on their history of body condition 15 weeks before calving, to form two groups, one with normal (NBCS) and one with high BCS (HBCS). Until drying-off, the two groups were fed with diets differing in energy content for promoting the difference in BCS until dry-off. Thereafter all cows were fed the same diets.

Using this experimental approach, we hypothesized that (a) the differences in body condition will be maintained between the groups during the transition into the next lactation, (b) HBCS would mobilize more lipid reserves than NBCS cows and have greater milk fat contents.

Besides expecting elevated concentrations of NEFA, ß-hydroxybutyrate (BHB), and leptin in serum of HBCS cows, we also hypothesized that (c) HBCS cows would have lower concentrations of insulin, IGF-1, and adiponectin, and also experience more oxidative stress than NBCS cows during early lactation. Moreover, based on reports about leptin-linked increased levels of thyroid hormones in obese as compared to normal-weight human patients (Reinehr, 2010), we hypothesized that (d) HBCS cows might have elevated thyroid hormone concentrations around parturition.

## Materials and methods

The described animal experiment was conducted at the experimental station of the Educational and Research Centre for Animal Husbandry, Hofgut Neumuehle, Muenchweiler a. d. Alsenz, Germany. The study covered a period over 29 weeks, starting 15 weeks before the anticipated calving date and ending 14 weeks thereafter. Blood sampling was limited to 7 weeks *antepartum (a.p.)* until 12 weeks *post partum (p.p.)*.

### *Animals and Feeding Regimen*

Thirty-eight pregnant multiparous German Holstein dairy cows (average parity: 2.9 ± 0.3, mean ± SEM) were allocated 15 weeks before their expected calving date to either the HBCS (n = 19) or the NBCS (n = 19) group. These two groups were fed differently during late lactation as detailed below to reach different targets for BCS and back fat thickness (BFT) at dry-off (HBCS: > 3.75 and > 1.4 cm; NBCS: <3.5 and <1.2 cm). The BCS was estimated on a 5-point scale, whereas BFT was assessed in the sacral region using ultra-sonography (AGROSCAN L, ALR 500, 5 MHz, linear-array transducer, Echo Control Medical, Angoulême, France).

Both BCS and BFT were continuously monitored biweekly (week 15 *a.p.* to week 15 *p.p.*) by one person. The two groups were initially pre-selected from the entire herd (150 lactating cows) by their history of body condition, that is, using BCS and BFT records from the preceding lactation. For this, the BCS and BFT records from all cows at the experimental farm during the year preceding the trial were considered to find cows divergent in both variables for forming two groups with equal numbers. The cows were classified as HBCS cows when mean BFT around the preceding calving was > 1.2 cm or maximal BFT during lactation was ≥ 1.9 cm and mean BCS > 3.2 or maximum BCS ≥ 3.75, respectively. The BFT and BCS values for the pre-selection of NBCS cows were below these limits.

The cows were also stratified for comparable 305-days milk yields from previous lactations (NBCS: 10 361 kg ± 302 kg; HBCS: 10 315 ± 437 kg, means ± SEM). After pre-selection, cows were allocated 15 weeks *a.p.* to two feeding groups (for the diets see Table 1) to accentuate the differences in body condition: NBCS animals were fed a low-energy ration [6.8 NEL (MJ/kg of DM)], whereas HBCS animals were fed the fresh cow ration with higher energy content [7.2 NEL (MJ/kg of DM)], from week 15 to 7 before the anticipated calving date. During the subsequent dry-off period, both groups received the same ration, followed by the same fresh-cow ration in lactation. All diets were fed as total mixed ration (TMR) consisting of 63% roughage and 37% concentrate in the high-energy ration, or 74% roughage and 26% concentrate in the low-energy ration. Samples of all individual components of the TMR as well as the concentrate feed were collected biweekly and stored at -20 °C until analysis. To determine the DM content, feed samples were dried at 60 °C for 24 h and then at 105 °C for 3 h. The nutrient composition of the feed samples was analyzed according to the official recommendations of the Association of German Agricultural Analytic and Research Institutes (Naumann and Bassler, 2004). Samples were analyzed for dry matter, crude ash, crude protein (CP), utilizable CP, crude fat, crude fiber, acid detergent fiber (ADF), neutral detergent fiber (NDF), and non-fiber carbohydrates (NFC) while the minerals calcium, phosphorus, magnesium, sodium and potassium were analyzed by x-ray fluorescence analysis.

The energy content of the diet (ME and NEL) was calculated according to the German Society of Nutrition Physiology (GfE, 2009). Ingredients and nutrient composition are shown in Table 1.

The rations were balanced to meet the nutritional requirements of cows according to the recommendations of the Society of Nutrition Physiology in Germany (GfE, 2001). Animals were housed in an open barn, with permanent free access to feed and water. The diet was provided twice daily while cows had access to several feeders. Individual daily feed intake was recorded from week 3 *a.p.* until week *14 p.p.* using an electronic feeding system (Roughage Intake Control System; Insentec B.V., Marknesse, Netherlands); due to the conditions at the research farm, earlier prepartum intake could not be recorded. From all cows, BW was determined by an electric scale on a weekly basis a.p., and twice daily p.p. after each milking. Cows were milked twice daily at 0500 and 1530 in a milking parlour (GEA Farm Technologies GmbH, Boenen, Germany).

**Table 2** Ingredient composition and chemical composition (% of dry matter (DM), unless otherwise noted) of rations during the observation period for cows of the high body condition score (HBCS) and normal body condition score (NBCS) group

| | Late lactation | | Dry period | Early lactation |
|---|---|---|---|---|
| | 15 to 7 weeks *a.p.* | | week 7 *a.p.* to parturition | 1 to 14 weeks in milk |
| Item | HBCS | NBCS | HBCS / NBCS | HBCS / NBCS |
| Ingredient | | | | |
| Grass silage | 22.4 | 32.0 | 32.0 | 22.4 |
| Corn silage | 20.7 | 32.0 | 32.0 | 20.7 |
| Pressed beet pulp silage | 12.5 | - | - | 12.5 |
| Hay | 5.5 | 5.4 | 5.4 | 5.5 |
| Straw | 2.3 | 4.1 | 4.1 | 2.3 |
| Vitamin and mineral mix[1] | 0.4 | 0.7 | 0.7 | 0.4 |
| Concentrate[2] | 36.2 | 25.8 | 25.8 | 36.2 |
| Analysed chemical composition | | | | |
| ME[4](MJ/kg of DM) | 10.8 | 10.6 | 10.6 | 10.8 |
| $NE_L$[5] (MJ/kg of DM) | 7.2 | 6.8 | 6.8 | 7.2 |
| Crude protein (g/kg of DM) | 170 | 157 | 157 | 170 |
| Utilizable CP (g/kg of DM) | 156 | 149 | 149 | 156 |
| NDF (g/kg of DM) | 359 | 382 | 382 | 359 |
| ADF (g/kg of DM) | 204 | 223 | 223 | 204 |
| NFC (g/kg of DM) | 402 | 360 | 402 | 360 |
| Ruminal N balance (g/d) | 3.4 | 2.3 | 2.3 | 3.4 |

*a.p. = Antepartum;* ME = metabolizable energy; $NE_L$ = net energy for lactation; NDF = neutral detergent fibre; ADF = acid detergent fibre; NFC = non-fibre carbohydrate.
[1]Provided per kilogram total mixed ration (on DM basis): calcium, 0.36 g; phosphorus, 0.36 g; sodium, 0.36 g; magnesium, 0.40 g; zinc, 28 mg; manganese, 17 mg; copper, 6.0 mg; cobalt, 0.24 mg; iodine, 0.80 mg; selenium, 0.21 mg; vitamin A, 4.000 IU, vitamin D, 600 IU, vitamin E, 20 mg (RINDAMIN K11 ATG, Schaumann, Pinneberg, Germany).
[2]Concentrate portion consisted of barley (25% of DM), corn grain (31% of DM), soybean meal (18% of DM), and canola meal (26% of DM).

The calculations for the net energy requirement for maintenance (NEM), pregnancy, and those for lactation (NEL), as well as the milk energy concentrations were made according to the guidelines of the Society of Nutrition Physiology (GfE, 2001) as follows:

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

Maintenance and pregnancy (6 to 4 weeks *a.p.*; MJ $NE_L$/d): $NE_M$ + 13;

Maintenance and pregnancy (3 weeks *a.p.* until calving; MJ $NE_L$/d): $NE_M$ + 18;

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

Energy requirement for lactation $NE_L$ (MJ $NE_L$/d) = [milk energy concentration (MJ $NE_L$/kg) + 0.086] x milk yield (kg/d);

Net energy balance (EB, MJ $NE_L$/d) = energy intake (MJ $NE_L$/d) – NEM (MJ $NE_L$/d) – $NE_L$ (MJ $NE_L$/d);

Energy intake = daily DMI x energy content of the TMR ($NE_L$/kg DM).

Energy-corrected milk (ECM) was calculated based on the equation of the German Agricultural Society (Deutsche Landwirtschaftsgesellschaft, 2000):

ECM (kg/d) = milk yield (kg/d) x [1.05 + (milk fat (%) × 0.38 + milk protein (%) × 0.21)]/3.28.

*Analyses in Milk and Blood Samples*

Proportional milk samples were collected weekly until 14 weeks *p.p.* and pooled from two consecutive milkings (0500 and 1530; 50:50 vol/vol).

Milk fat, protein, lactose, urea, and somatic cell counts were assessed using a milk analyzer based on Fourier transform infrared spectroscopy (Bentley FTS, Bentley Instruments, Inc., Chaska, Minnesota, USA) at the laboratory of the milk recording organization, Milchprüfring Baden-Württemberg e.V., Kirchheim, Germany.

In addition, from week 7 *a.p.* until week 12 *p.p.*, blood was collected weekly from the V. coccygea with S-Monovettes® (Sarstedt, Nümbrecht, Germany), after the morning milking but before providing fresh feed. Blood samples were kept at room temperature until coagulated (max. 60 min), centrifuged for 10 min at 2000 × **g** and subsequently stored at -20 °C until analysis. Serum concentrations of NEFA, BHB, glucose, leptin, haptoglobin, adiponectin, derivatives of reactive oxygen metabolites (dROM) and total ferric reducing antioxidant power (FRAP) were analysed weekly, whereby leptin measurements were limited to the time from week 7 a.p. until week 5 p.p., and to week 12 p.p. Serum BHB, glucose, and NEFA were measured at the Institute of Animal Nutrition, Friedrich-Loeffler-Institute (FLI) in Braunschweig, Germany, using an automatic photometric analysing system (Eurolyser, Type VET CCA, Salzburg, Austria).

Leptin, haptoglobin and adiponectin were measured by in-house developed ELISAs (Sauerwein et al., 2004; Hiss et al., 2009; Mielenz et al., 2013). For the leptin ELISA, the intra- and inter-assay coefficient of variations (CV) were 6.3 and 13.9%, the standard curve reached from 0.11 – 27 ng/mL, and the measuring range was 0.3 – 7 ng/mL. The corresponding numbers for haptoglobin were 3.9 and 12.2%, the range of the standard curve was 0.012 – 9 µg/mL with a measuring range of 0.1 to 2 ug/mL, and for adiponectin 4.5 and 5.6%, with a standard curve ranging from 0.019 to 20 ng/mL, and a measuring range of 0.3 7 ng/mL, respectively. Serum dROM were measured using N,N-diethyl-para-phenylendiamine (DEPPD) as chromogene with the modifications of Regenhard et al. (2014); results are given as H2O2 equivalents; the intra and interassay CV were 6.3 and 10.0%, respectively.

Total ferric reducing antioxidant power (FRAP) was measured according to Benzie and Strain (1996), as the ability of serum to reduce Fe3+ (FeCl3•6 H2O) to Fe2+; values are given as µmol Fe2+/L. The intra- and inter-assay CV were 2.7 and 2.6 %.

Thyroid hormone concentrations, free 3-3′-5-triiodothyronine (fT3) and free thyroxine (fT4), were analysed in weeks 7, 3, and 1 a.p. as well as in weeks 1, 2, 3, 5, 7, 9, and 12 p.p. at the Central Laboratory of the University Hospital in Bonn, Institute of Clinical Chemistry and Clinical Pharmacology, by electro-chemiluminescent immunoassay (ELICA; Roche Diagnosis GmbH, Mannheim, Germany). Circulating insulin and IGF 1 were analysed in weeks 7 and 2 *a.p.* and in weeks 1 and 4 *p.p.* at the clinic for cattle, University of Veterinary Medicine (TiHo) Hannover.

For IGF-1, a radioimmunoassay (RIA) was used (A15729, IGF-I IRMA; Immunotech, Beckman Coulter, Brea, CA, USA). The intra- and inter-assay CV were 5.1 and 9.3%, respectively, the limit of detection (LOD) was 33 ng/mL. Insulin concentrations were determined via RIA (IM3210, Insulin IRMA KIT, Immunotech, Beckman Coulter, Brea, CA, USA). The intra- and inter-assay CV were 7.6 and 10.7%, respectively, the LOD was 3 µU/mL.

The threshold concentrations of BHB in serum used for defining hyperketonaemia or subclinical ketosis (SCK) were > 1.2 mM and > 2.5 mM for clinical ketosis (CK), respectively (Schulz et al., 2014).

*Statistical Analyses*

Statistical analysis of the data was carried out using SPSS software (IBM® SPSS® Statistics 24.0). Data were analysed using the mixed model ANOVA with repeated measurements. The

Bonferroni correction method was used for correction of multiple comparisons. The mixed models used contained the fixed effects of treatment (group), time (weeks relative to calving), and the interaction between treatment and time, while the individual "cow" was considered as a random factor. Lactation number was considered as a covariate. When insignificant it was excluded from the model. The level of significance was set at $P \leq 0.05$ and a trend was defined at $0.05 < P \leq 0.10$.

The residuals of each variable were tested for normal distribution. For mixed model analyses, data were transformed by a two-step approach to become normally distributed as described by Templeton (2011). In step 1, variables were transformed into a percentile rank, resulting in uniformly distributed probabilities. In step 2, results from the first step were inverse-normal transformed, creating variables consisting of normally distributed z-scores. For all graphs, non-transformed data (means ± SEM) were used. Relationships between variables were tested by Spearman correlation. Potential associations were tested for the periods before and after parturition, as well as for the whole experimental period. Only correlations with $r > 0.4$ and $P < 0.05$ are reported.

## Results and discussion

The general relationship between overcondition and risk for metabolic diseases, in particular ketosis, is known from both retrospective analyses of spontaneously developed overcondition (e.g., Smith *et al.*, 2017) and experimental over-conditioning of cows by feeding more energy-dense diets during either the entire dry period, or the far-off or the close-up phase of the dry period (e.g., Dann *et al.*, 2006). In some studies, in which different energy levels were tested during the dry period, cows were preselected based on their spontaneously developed BCS (e.g., Schulz *et al*., 2014), or were target-fed before drying off to achieve groups differing in BSC (Roche *et al*., 2013 and 2015). The latter approach is similar to the one taken herein, except that we did a preselection of the pluriparous cows according to their spontaneously developed BCS well before dry off, and limited the time of differential feeding to 8 weeks before drying off.

### *Performance in high- and normal-conditioned cows*

The variables describing body condition and energy status (BCS, BFT, DMI, and EB) in HBCS and NBCS cows are presented in Figures 1a to d. The classification according to BCS and BFT 15 weeks *a.p.* yielded initial differences of about 0.4 BCS points and 0.5 cm BFT. Feeding different energy levels from 15 weeks *a.p.* until dry-off augmented the differences to 0.8 BCS

units and 1.1 cm BFT in week 7 *a.p.* The targeted BCS and BFT at dry-off (HBCS: > 3.75 and > 1.4 cm; NBCS: <3.5 and <1.2 cm) were thus achieved. During the dry period, when both groups received the same diets, they increased their body condition whereby the previously established differences were largely maintained until the week before calving (Δ = 0.7 BCS points and 1.1 cm BFT). Body condition declined during lactation in both groups, but the losses were bigger in the HBCS than in the NBCS cows.

At the end of the observation period in week 15, the difference between the groups was about the same as at the initial grouping in the preceding lactation. For explaining the divergent development of body condition in individual cows kept under the same management and feeding conditions, genetic predisposition as well as feed intake, milk yield, and feed conversion ratio likely play a role (Rocco and McNamara, 2013).

Feed intake data recorded *a.p.* in our study were limited to the last 3 weeks before calving; intake was greater in NBCS than in HBCS cows until calving when both groups reached the same nadir 1-week *p.p.* During the subsequent weeks NBCS cows had a faster increase in feed intake; the difference between groups levelled off in week 11 *p.p.*

The calculated EB was higher in NBCS than in HBCS cows *a.p.* and also reached positive values about 2 weeks earlier than in the HBCS group. These differences were rather attributable to feed intake than milk yield since neither milk nor ECM yield differed between the groups. However, there was a group by time interaction for milk yield and a trend for such an interaction for ECM. The NBCS tended to have greater yields during the first 4 weeks of lactation; thereafter the yield curves were approximately at the same level (Figure 2a and b).

The 100-d milk yield (weeks 1 to 14) was also the same in both groups (HBCS: 3,816 ± 114 kg; NBCS: 3,875 ± 93 kg). With the exception of lactose, milk composition including urea, and also protein and fat yield were not different between the groups in general (Figure 2c, d and f); for lactose the concentrations tended to be greater in NBCS cows as well, in particular during the first 5 weeks *p.p.* (Figure 2e). These results are contrary to several reports in the literature showing that milk yield, partly including also protein, fat and lactose yields, increased with BCS (e.g., Roche *et al.*, 2009, 2013, and 2015).

The reason for the contradicting results might be attributable to different feeding and management conditions (e.g. many of the aforementioned studies were done in pasture-based systems), and also to the absolute range of BCS achieved in our HBCS animals: Roche *et al.* (2007) pointed out that the increase in milk yield and in fat corrected milk was getting

smaller with BCS ≥ 3.0 at calving. However, elevated BCS was also reported to result in reduced milk production (Roche *et al.*, 2009).

Taking together, the mostly insignificant results for yields, the HBCS cows albeit eating less than NBSC cows, were able to maintain milk performance at a similar level as the NBCS cows, likely by the greater mobilization of body reserves compared to NBCS cows.

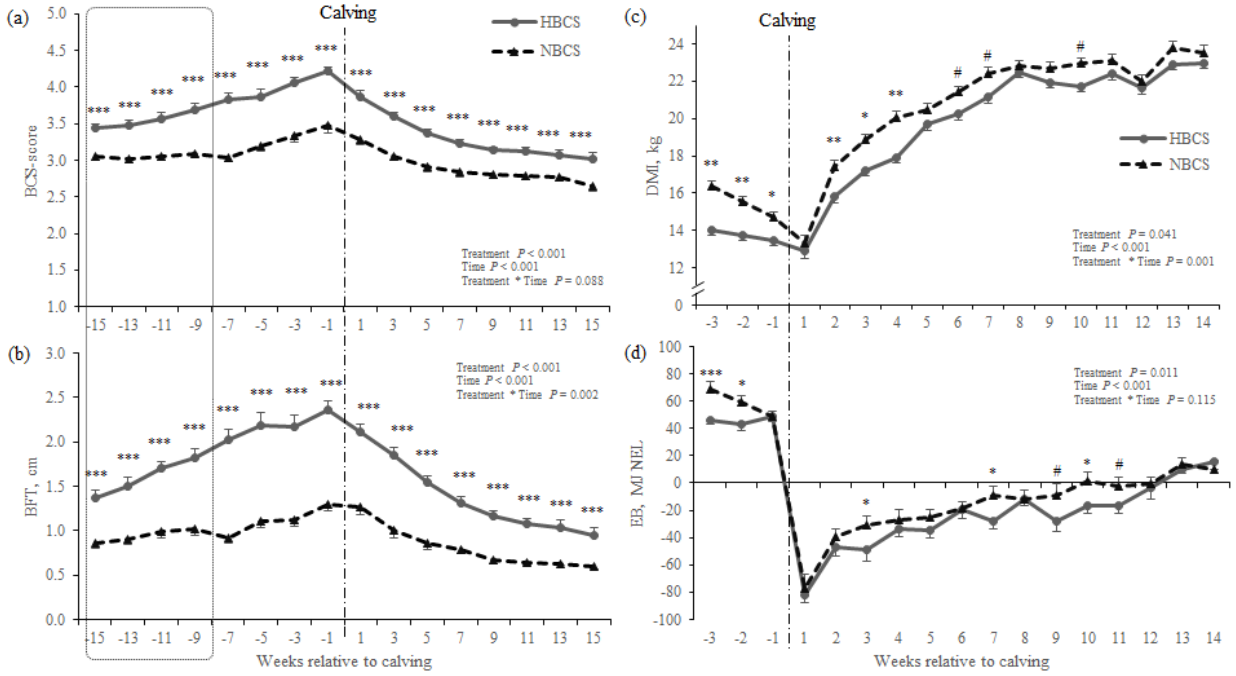


**Figure 1** Changes of (a) body condition score (BCS) and (b) back fat thickness (BFT) from 15 weeks *antepartum (a.p.)* to 15 weeks *post partum* (*p.p.)* as well as (c) dry matter intake (DMI) and (d) energy balance (EB) from 3 weeks *a.p.* until 14 weeks *p.p.* (time = weeks relative to calving) in high BCS (HBSC) or normal BCS (NBCS) cows. The area framed by dotted lines indicates the time of differential feeding of HBCS and NBCS cows. The vertical dashed line illustrates calving. Results are presented as means ± SEM. Significant differences between the groups are indicated with asterisks (*) when $P \leq 0.05$, or (**) when $P \leq 0.01$, or (***) when $P \leq 0.001$ at a given time point, respectively. Trends ($P \leq 0.10$) for differences between the groups at a given time point are indicated by (#).

*Serum Metabolites*

*Concentrations of non-esterified fatty acids, ß-hydroxybutyrate and glucose.* The NEFA concentrations tend to increase during late gestation due to reduced feed intake (Bell, 1995) at a time when foetal growth reaches its exponential phase. Moreover, when nutrient intake cannot meet the requirements for the increasing demands also for the mammary gland, body reserves, mainly from adipose tissue, are mobilized to compensate the lack of energy intake. Expectedly, the circulating NEFA concentrations increased towards calving and were further elevated during lactation (Figure 3a). The concentrations in the HBCS group increased earlier and to greater levels than in the NBCS group indicating that lipolysis was more

pronounced than in NBCS cows. Positive correlations between the NEFA concentrations and BFT *p.p.* ($r$ = 0.456; $P$ < 0.001) and negative ones with DMI and EB ($r$ = - 0.491 and $r$ = - 0.469, respectively; $P$ < 0.001) were observed.

The uptake of NEFA by the mammary gland for milk fat synthesis is greatest at the onset of lactation; in later stages *de novo* synthesis of fatty acids increases (Bell, 1995). However, as pointed out above, the greater NEFA circulating concentrations in HBCS cows did not result in significant quantitative changes of milk fat content or yield.

In phases of energy deficit, NEFA are only incompletely oxidized to acetyl-CoA and serve ketogenesis including the production of BHB. However, NEFA as well as ketone bodies may also provide energy for tissues, other than the mammary gland (Drackley *et al.*, 2001). As indicated by the group x time interaction, the time course of the BHB concentrations in HBCS cows was different from the one in NBCS cows: the *postpartal* increase was largely limited to HBCS cows (Figure 3b).

In addition, hyperketonaemia (BHB > 1.2 mmol/l) was more frequent in HBCS cows (HBCS cows: 83% *versus* NBCS cows: 61%) and also lasted longer compared to NBCS cows. These observations seem to be in line with the lesser DMI in HBCS cows, since it is probable that increased hepatic fatty acid oxidation, as a consequence of plasma NEFA and hepatic fatty acid uptake, created a satiety signal in these cows according to hepatic oxidation theory (Allen *et al.*, 2009). For glucose, slightly greater (~ 15%) circulating concentrations were observed in HBCS cows compared to the NBCS group both *a.p.* and *p.p.* (Figure 3c). With the onset of lactation, the requirements for glucose rapidly increase to serve lactose production (Bell, 1995). The use of glucose in other peripheral tissues is concomitantly decreased (Bell, 1995). Increased body condition before calving was reported to be associated with greater blood glucose concentrations, suggesting that less glucose was used for milk production in cows with higher BCS (Dechow *et al.*, 2017). The mammary uptake of glucose was shown to be independent of the arterial concentrations (Nielsen *et al*., 2001) and greater circulating glucose but lower milk lactose concentrations in HBCS cows in our study are in line with this. Both ketones and NEFA can be used as energy source by various tissues in the body including the mammary gland in favour of milk production (Drackley *et al.*, 2001) and thus may explain why milk yield was not compromised in HBCS cows.

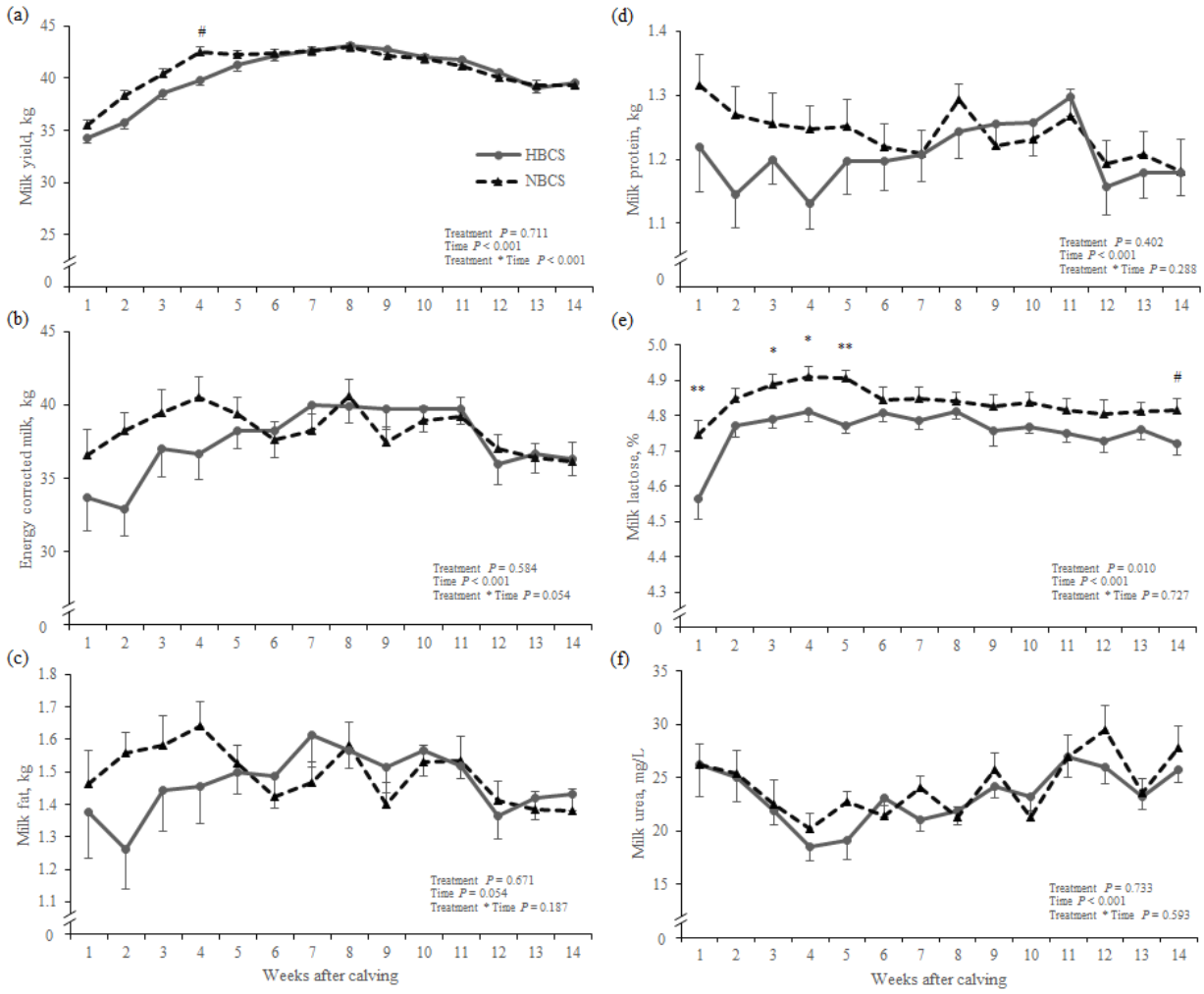


**Figure 2** Yields of (a) milk, (b) energy-corrected milk, (c) milk fat and (d) milk protein, and concentrations of (e) lactose and (f) urea in milk in high body condition score (HBCS) or normal body condition score (NBCS) cows from 1 to 14 weeks *post partum* (time = weeks relative to calving). Results are presented as means ± SEM. Significant differences between the groups are indicated with asterisks (*) when P ≤ 0.05 or (**) when P ≤ 0.01 at a given time point, respectively. Trends (P ≤ 0.10) for differences between the groups at a given time point are indicated by (#).

*Variables indicative for the oxidative status.* Reactive oxygen metabolites in serum indicate elevated production of free radicals or a decreased antioxidant protection. The values of dROM changed with time, but were not different between the two BCS groups (Figure 3d). Numerically higher values were observed for HBCS cows after calving compared to NBCS cows and may thus considered to be in line with earlier findings that cows with greater BCS and pronounced BCS losses around calving had also greater dROM values (Bernabucci *et al*., 2005).

When comparing the FRAP values, reflecting the antioxidative capacity, changes with time were similar in both groups with lowest values before calving, but the HBCS cows had lower values (Figure 3e). The increasing output of antioxidants via colostrum together with the decreasing input with feed likely explains for the time course. The more pronounced depression in DMI of the HBCS might account, at least partly, for the difference between the two BCS groups.

*Metabolic hormones assessed in serum.* In both groups, insulin and IGF-1 had lower concentrations in lactation than in pregnancy (Figure 4a and b). This is in line with the typical hypoinsulinaemia in early lactation which decreases lipogenesis, promotes lipolysis, and reduces glucose uptake by peripheral tissues thus facilitating the insulin-independent mammary glucose uptake (Bell, 1995). Hypoinsuliaemia is also related to the uncoupling of the somatotropic axis which in turn leads to decreased secretion of IGF-1 (Butler *et al.*, 2003). When considering all time points, HBCS cows had greater insulin concentrations than NBCS cows, but differences could not be assigned to individual time points when doing Bonferroni-corrected multiple comparisons. Greater insulin but also glucose concentrations in HBCS cows indicate decreased insulin sensitivity (**IS**). The notion that IS decreases with BCS is quite common, but largely relies on surrogate indices for insulin sensitivity and not on clamp studies considered as "gold standard" for assessing IS. However, the latter, performed in dry or late lactating cows are in support of decreasing IS with increased BCS (e.g. de Koster *et al.*, 2015). In our study, the insulin concentrations were correlated with glucose ($r = 0.464$; $P < 0.001$), IGF-1 ($r = 0.658$; $P < 0.001$), NEFA ($r = -0.579$; $P < 0.001$) and with leptin ($r = 0.517$; $P < 0.001$). The IGF-1 concentrations in serum were correlated to the EB ($r = 0.721$; $P < 0.001$), NEFA ($r = -0.612$; $P < 0.001$) and also with leptin ($r = 0.435$; $P < 0.001$).

Leptin is involved in controlling energy homeostasis as well as feed intake and is positively associated with BCS, BW, and adipocyte size (Locher *et al.*, 2015). During the dry period, HBCS cows had up to 2.8-fold greater leptin concentrations than the NBCS cows (Figure 4c). The *antepartal* decrease of leptin started also about 2 weeks earlier in the HBCS than in the NBCS cows. Comparable results were reported by Kokkonen *et al.* (2005) with a more pronounced decrease of circulating leptin in high-mobilizing cows from the last week *a.p.* until the 1st week in milk; the leptin concentrations in the latter study also remained higher *p.p.* in fatter compared to thinner cows. As expected, circulating leptin was also correlated with BW and BCS ($r = 0.482$ and $r = 0.493$, respectively; $P < 0.001$).

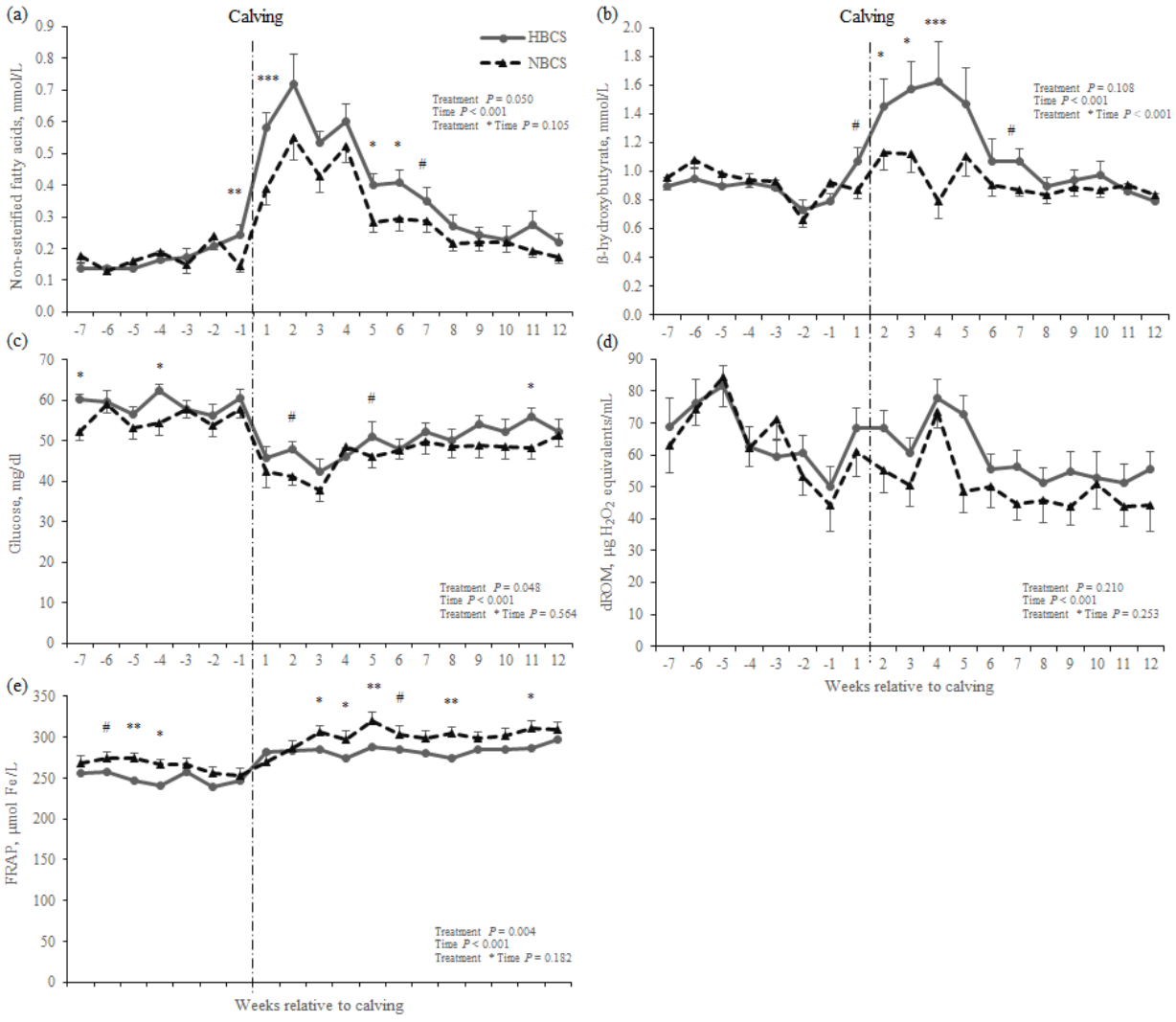


**Figure 3** Serum concentrations of (a) non-esterified fatty acids, (b) *ß*-hydroxybutyrate, (c) glucose, (d) derivatives of reactive oxygen metabolites (dROM) and (e) ferric reducing ability of plasma (FRAP) in high body condition score (HBCS) or normal body condition score (NBCS) cows from 7 weeks *antepartum* to 12 weeks *post partum* (time = weeks relative to calving). Results are presented as means ± SEM. Significant differences between the groups are indicated with asterisks (*) when $P \leq 0.05$, or (**) when $P \leq 0.01$, or (***) when $P \leq 0.001$ at a given time point, respectively. Trends ($P \leq 0.10$) for differences between the groups at a given time point are indicated by (#).

Adiponectin is known for its insulin sensitizing effects and in line with this, its circulating concentrations during the transition phase of dairy cows decrease towards calving and reach lowest values during the 1st weeks of lactation (Sauerwein and Häußler, 2016).

This time course was also observed in the present study (Figure 4d). As indicated by the time by group interaction, the curves of HBCS and NBCS curves were not parallel but crossed: *a.p.* the greater values were mostly observed in HBCS values but *p.p.*, the values of the NBCS group exceeded those of the HBCS cows. The potential underlying mechanisms for the time course in general and the interaction in particular are largely unknown.

A comprehensive study testing different potential effectors of circulating adiponectin in dairy cows, yielded EB as a regulator, but neither lipid mobilization nor sustained changes in insulin, growth hormone, leptin, or fatty acids affecting adiponectin (Krumm *et al.*, 2017). It is well established that thyroid hormone status correlates with body weight and basal metabolic rate. The thyroid hormones T4 and T3 are secreted by the thyroid gland; T3 is also peripherally generated by deiodination of T4. Body fat content and thyroid status could be linked via leptin, since leptin concentrations are related to the release of thyroid stimulating hormone (TSH) (Reinehr, 2010). In our study, the fT4 concentrations in serum were indeed positively correlated with circulating leptin ($r$ = 0.547; $P$ < 0.001) providing some support for a relationship between leptin and thyroid status. However, we did not assess TSH in our study. The changes we observed with time for T3 and T4 largely correspond to previous reports (Nowroozi-Asl *et al.*, 2016); for T4, the *peripartal* decrease was more pronounced in our study than in the one from Nowroozi-Asl *et al*. (2016). The fT3 concentrations were not different between the groups (Figure 5a). For fT4 as well as for ratio fT3/fT4 time by group interactions were observed: HBCS cows had greater fT4 concentrations and a lower fT3/fT4 ratio than NBCS cows a.p., whereas p.p. the difference in fT4 had disappeared and the ratio fT3/fT4 was greater in the HBCS than in the NBCS cows. Albeit we observed no group effect for fT4, we found positive correlations between fT4 and BW ($r$ = 0.448; $P$ < 0.001), and EB ($r$ = 0.479; $P$ < 0.001). An increased fT3/fT4 ratio was shown to be associated with an increased risk of metabolic syndrome and insulin resistance in humans (Park *et al*., 2017).

However, taken together the data obtained for fT3, fT4 or fT3/fT4 in our study do not allow for a conclusive interpretation since the differences between groups were only small and mostly insignificant.

*Haptoglobin*. Parturition is related to inflammatory processes and acute phase proteins like Haptoglobin (Hp) are increased in the circulation around calving (Hachenberg *et al.*, 2007). Haptoglobin is mainly produced by the liver, but is also expressed in adipose tissue, undergoing similar changes as hepatic mRNA abundance and the circulating concentrations (Saremi *et al.*, 2012). This time course was also observed in our study and without group differences (Fig 5 D). The potential contribution of visceral and subcutaneous fat to the circulating concentration was estimated to amount to only 0.02% of the hepatic one (Saremi *et al.*, 2012).

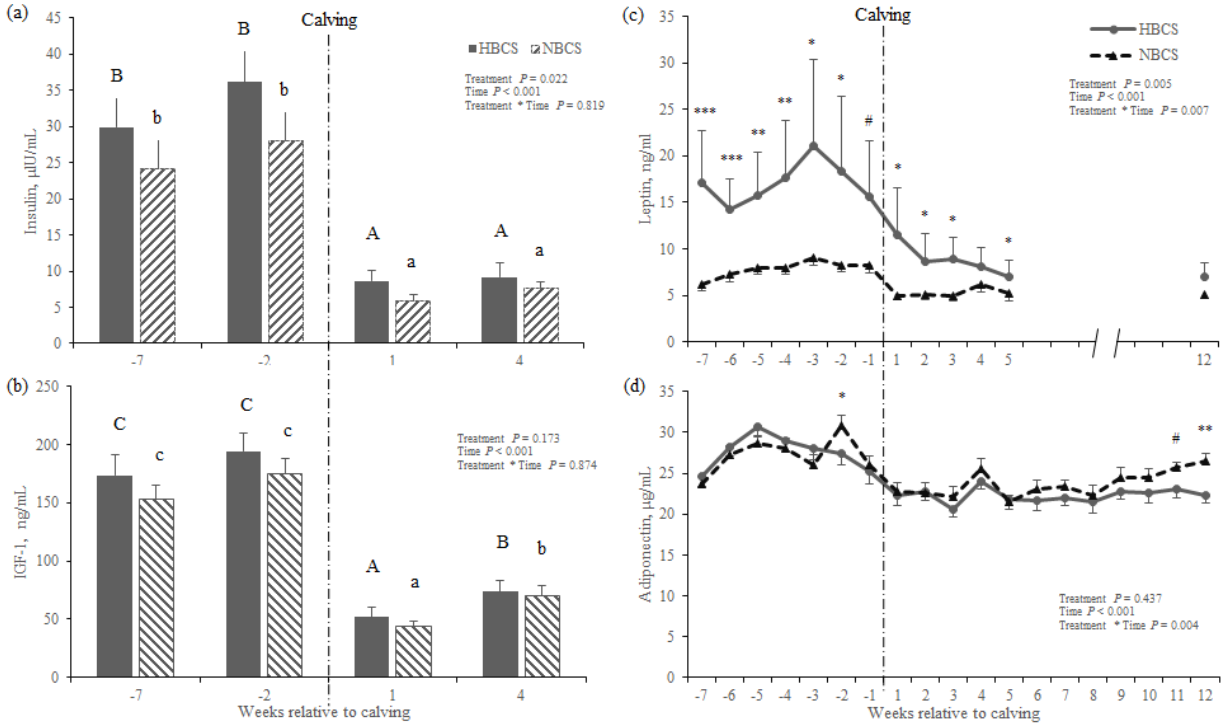


**Figure 4** Serum concentrations of (a) insulin, (b) IGF-1, (c) leptin and (d) adiponectin in high body condition score (HBCS) or normal body condition score (NBCS) cows (time = weeks relative to calving). Results are presented as means ± SEM. Significant differences between the groups are indicated with asterisks (*) when $P \leq 0.05$, or (**) when $P \leq 0.01$, or (***) when $P \leq 0.001$ at a given time point, respectively. Trends ($P \leq 0.10$) for differences between the groups at a given time point are indicated by (#). [A, B]Different capital letters indicate differences between the time points in the HBCS cows. [a, b]Different lowercase letters stand for differences between the time points in the NBCS cows.

Indeed, when grouping cows according to their BSC, or the extent of the BCS loss from 2 weeks *a.p.* to 4 weeks *p.p.*, Hachenberg *et al.* (2007) found no differences in circulating Hp. Reports about associations of Hp with NEFA or BHB are inconsistent: some studies showed positive correlations (e.g., Hiss *et al.*, 2009), others did not (e.g., Hachenberg *et al.*, 2007). In the present study Hp was not correlated with BHB and only weakly with NEFA (r = 0.24; $P \leq 0.05$).

Negative correlations were observed with insulin ($r$ = - 0.486; $P < 0.001$) and IGF-1 ($r$ = - 0.712; $P < 0.001$), respectively. However, the individual Hp concentrations showed considerable variation, in particular 6 to 3 weeks *a.p.* in our study, with numerically higher concentrations in NBCS cows compared to HBCS cows. There were no clinical signs recorded in the animals with elevated Hp and thus the reasons for the variation remain unexplained.

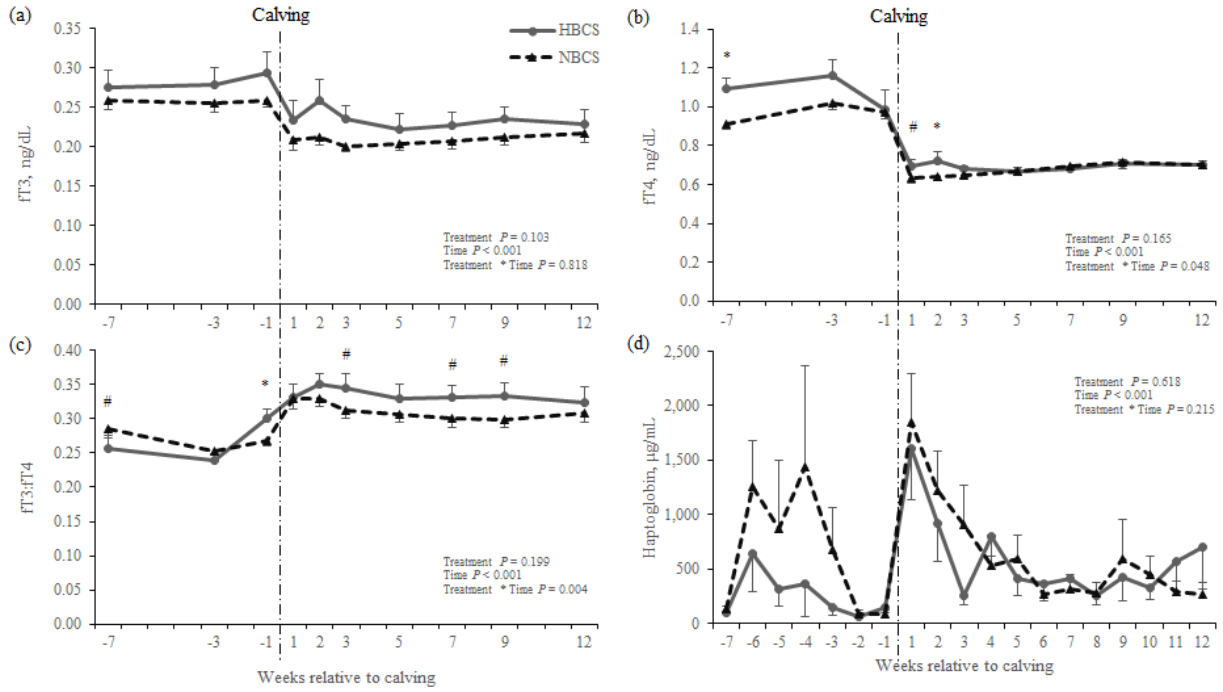


**Figure 5** Serum concentrations of (a) free triiodothyronine (fT3), (b) free thyroxine (fT4), (c) the ratio fT3/fT4 and of (d) haptoglobin in high body condition score (HBCS) or normal body condition score (NBCS) cows (time = weeks relative to calving). Results are presented as means ± SEM. Significant differences between the groups are indicated with asterisks (*) when $P \leq 0.05$ at a given time point. Trends ($P \leq 0.10$) for differences between the groups at a given time point are indicated by (#).

## Conclusion

The experimental approach taken yielded cows differing in BCS at dry off and maintaining this difference until calving and over 14 weeks of lactation. Cows calving with high BCS were metabolically challenged during early lactation due to a more sever negative EB and intense mobilization of body fat, associated with reduced early lactation DMI. In addition, high BCS at calving was associated with compromised antioxidative capacity, reflected by lower values of FRAP. In contrast to our hypothesis, HBCS cows had greater insulin concentrations than NBCS cows, accompanied by greater glucose concentrations which may indicate reduced IS in HBCS cows. The serum concentrations of IGF-1 were not affected by overconditioning, but were lower in lactation than in pregnancy in both groups. The HBCS cows had greater concentrations of leptin than NBCS cows.

Cows calving with high BCS had elevated serum fT4 concentrations and a lower fT3/fT4 ratio than NBCS cow *a.p.*, whereas *p.p.* ratio of fT3/fT4 followed a reverse pattern as that of *a.p*. Together, the differences in BCS were accompanied with concomitant changes in blood metabolites and hormones thus confirming the adequacy of the animal model for studying different intensities of mobilization.

**Acknowledgements**

K. Schuh is recipient of a scholarship from the H. Wilhelm Schaumann Foundation (Hamburg, Germany). The authors thank M. Schmicke, University of Veterinary Medicine in Hannover, for providing the results of insulin and IGF-1 concentrations, assessed in the frame of the nation-wide German project "Opti-Kuh" financed through the Innovation Fund of the Federal Ministry for Nutrition and Agriculture (BMEL). For their excellent technical assistance and support the authors also thank B. Heitkönig, T. Blees, and K. Strack. The study would not have been possible without the Educational and Research Centre for Animal Husbandry, Hofgut Neumuehle since they made the cows available and also supported the animal trial by their staff.

**Declaration of interest**

The authors declare that they have no conflict of interest.

**Ethics committee**

The animal trial was approved by the local authority for animal welfare affairs (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany [G 14-20-071]).

**Software and data repository resources**

None of the data were deposited in an official repository.

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## 4 Manuscript II

*(submitted and under review)*

# Blood and adipose tissue steroid metabolomics and mRNA expression of steroidogenic enzymes in periparturient dairy cows differing in body condition

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**Abstract**

In high-yielding dairy cows, the rapidly increasing milk production after parturition can result in a negative nutrient balance, since feed intake is insufficient to cover the needs for lactation. Mobilizing body reserves, mainly adipose tissue (AT), might affect steroid metabolism. We hypothesized, that cows differing in the extent of periparturient lipomobilization, will have divergent steroid profiles measured in serum and subcutaneous (sc)AT by a targeted metabolomics approach and steroidogenic enzyme profiles in scAT and liver. Fifteen weeks antepartum, 38 multiparous Holstein cows were allocated to a high (HBCS) or normal body condition (NBCS) group fed differently until week 7 antepartum to either increase (HBCS BCS: 3.8 ± 0.1 and BFT: 2.0 ± 0.1 cm; mean ± SEM) or maintain BCS (NBCS BCS: 3.0 ± 0.1 and BFT: 0.9 ± 0.1 cm). Blood samples, liver, and scAT biopsies were collected at week -7, 1, 3, and 12 relative to parturition. Greater serum concentrations of progesterone, androsterone, and aldosterone in HBCS compared to NBCS cows after parturition, might be attributed to the increased mobilization of AT. Greater glucocorticoid concentrations in scAT after parturition in NBCS cows might either influence local lipogenesis by differentiation of preadipocytes into mature adipocytes and/or inflammatory response.

**Key words:** adipose tissue mobilization, periparturient period, cattle, steroid metabolomics, steroidogenic enzymes

**Introduction**

The periparturient period in high-yielding dairy cows is associated with extensive physiological and metabolic adaptations. With the onset of lactation, the energy requirements for milk synthesis increase within a short period, during which the energy intake is commonly insufficient to meet the energy demands of the animals. Consequently, energy stores, mainly fat from adipose tissue (AT), are mobilized[1]. Adipose tissue is a highly active metabolic and endocrine organ, secreting hormones and cytokines into the circulation[2,3]. Due to their lipophilic character, steroid hormones can be stored and further metabolized by steroidogenic enzymes expressed in AT, thus modulating local steroid concentrations[3–5]. Moreover, adipocytes have the potential to synthesize steroids *de novo* from cholesterol and its precursors[6]. From the precursor steroids dehydroepiandrosterone

(DHEA) and DHEA-sulfate (DHEA-S) steroidogenic enzymes such as 3 β-hydroxysteroid dehydrogenases (HSD3) and 17 ß-hydroxysteroid dehydrogenases (HSD17) locally synthesize androgens and/or estrogens[7,8]. The enzyme aromatase (CYP19) generates estrogens from androgens, i.e. from androstenedione and testosterone. Besides transforming estradiol to estrone, the enzyme HSD17 type 12 (HSD17B12) is involved in the elongation processes of very-long-chain fatty acids (VLCFA)[9]. Furthermore, HSD17B12 is highly expressed in organs related to fatty acid (FA) synthesis[10]. The steroidogenic acute regulatory protein (StAR) triggers cholesterol delivery to the inner mitochondrial membrane[6]. From there, the cholesterol side-chain cleavage enzyme (CYP11A1, also known as P450scc) initiates steroidogenesis by converting cholesterol to pregnenolone, acting as a precursor for all endogenous steroids[11]. The biosynthetic pathways for progestagens and gestagens, gluco- and mineralocorticoids, androgens, and estrogens are shown in Figure 1 (main enzymes involved in steroid biosynthesis considered in the present study are highlighted within the pathways).

In humans, steroidogenic enzymes are involved in processes regulating obesity and central fat accumulation[3]. Moreover, sex steroids such as estrogens and androgens can participate in the regulation of body fat distribution and can locally influence AT function[12]. Newell-Fugate[13] reviewed several effects of steroids, in particular of androgens and estrogens, on AT functions such as lipogenesis, lipolysis, adipocyte differentiation, insulin sensitivity as well as on adipokine secretion, mainly in monogastric species. In high-yielding dairy cows, an elevated body condition score (BCS > 3.5) before parturition is associated with increased lipolysis and ketogenesis[14] as well as increased incidence of metabolic disorders during the transition period[1]. Throughout lipomobilization, not only FA, but also steroids can be released from AT into the circulation[15,16], contributing to the whole body`s steroid level. So far, studies of circulating steroids in dairy cows were mainly focused on fertility and reproduction; however, the amount of steroids synthesized and metabolized in bovine AT as well as their relation with the circulating concentrations has not been investigated. Moreover, regarding the putative relevance of steroids in AT, studies addressing the transition period in context with increased lipomobilization due to different body conditions of dairy cows are lacking to our knowledge.

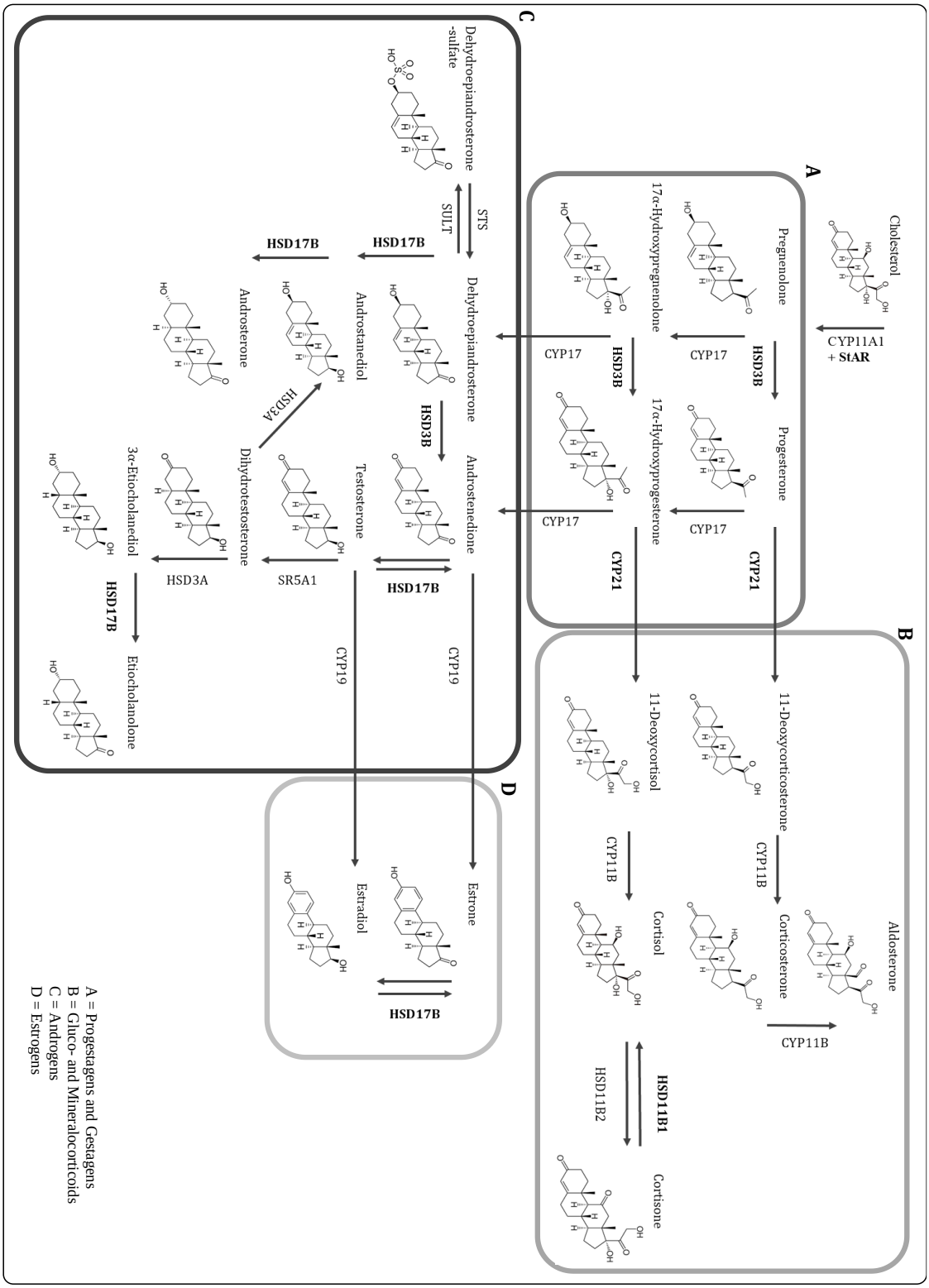


**Figure 1.** Biosynthetic pathway for (A) progestagens, (B) gluco- and mineralocorticoids, (C) androgens, and (D) estrogens. Enzymes involved in the steroidogenic pathway are shown next to the arrows, those enzymes being detected in the present study were highlighted in bold (the Figure was adapted from Figure 1 in MacKenzie et al.[60]. CYP11A1: cholesterol monooxygenase, CYP11B2: aldosterone synthase, CYP17: steroid-17⍰-hydroxylase, CYP19: aromatase, CYP21: steroid 21-hydroxylase, HSD3A: 3α-hydroxysteroid dehydrogenase, HSD3B: 3β-hydroxysteroid dehydrogenase, HSD11B1: 11ß-hydroxysteroid dehydrogenase type 1, HSD11B2: 11 ß-hydroxysteroid dehydrogenase type 2, HSD17B12: 17ß-hydroxysteroid dehydrogenase type 12, SR5A1: steroid-5α-reductase, StAR: steroidogenic acute regulatory protein, STS: steroid sulfatase, SULT: sulfotransferase.

In this study, we used an experimental model for dairy cows investigating high versus normal body tissue mobilization during the transition from pregnancy to lactation. Based on a targeted divergence in body condition in late lactation[14] we aimed to investigate the impact of body condition and lipomobilization on circulating and on AT specific steroid profiles from normal and over-conditioned cows around calving. We hypothesized that cows being distinct in the extent of periparturient lipomobilization, will also differ in their steroid and steroidogenic enzyme expression profiles. Thus, cows with a higher body condition around parturition will release more fat from AT and will have increased circulating steroid concentrations in peripheral blood when compared to cows with normal body condition. We aimed at characterizing the mRNA abundance of major steroidogenic enzymes in AT and liver and to compare the steroid hormone profiles in blood serum and in AT assessed by a targeted metabolomics approach. The objectives of the present study were (1) to describe the concentrations of steroid hormones, their precursors and metabolites in serum and subcutaneous AT (scAT) from late pregnancy through early lactation, (2) to assess the mRNA abundance of different key enzymes involved in steroid biosynthesis during the transition from late pregnancy to lactation in scAT and liver, and (3) to compare the steroid concentrations as well as the mRNA abundance of steroidogenic enzymes in dairy cows with high (HBCS) versus normal body condition score (NBCS) from late pregnancy through early lactation.

## Results

**Principal component analysis (PCA) and two-way heatmap clustering.** Applying PCA yielded significant separations between the steroids *ante partum* (a.p.) and *post partum* (p.p.) as well as for the steroid concentrations in serum and scAT (Figure 2A). However, the time pattern of steroid concentrations in scAT was comparable with steroids in serum. Using hierarchical cluster analysis, we clustered the steroids (with except for pregnenolone and pregnanediol) and presented it as a heat map (Figure 2B). The clustered heat map shows steroids in two main clusters. The first cluster contains glucocorticoids (cortisol, cortisone, and corticosterone) and the second cluster contains all other steroids (progestagens, androgens, and estrogens).

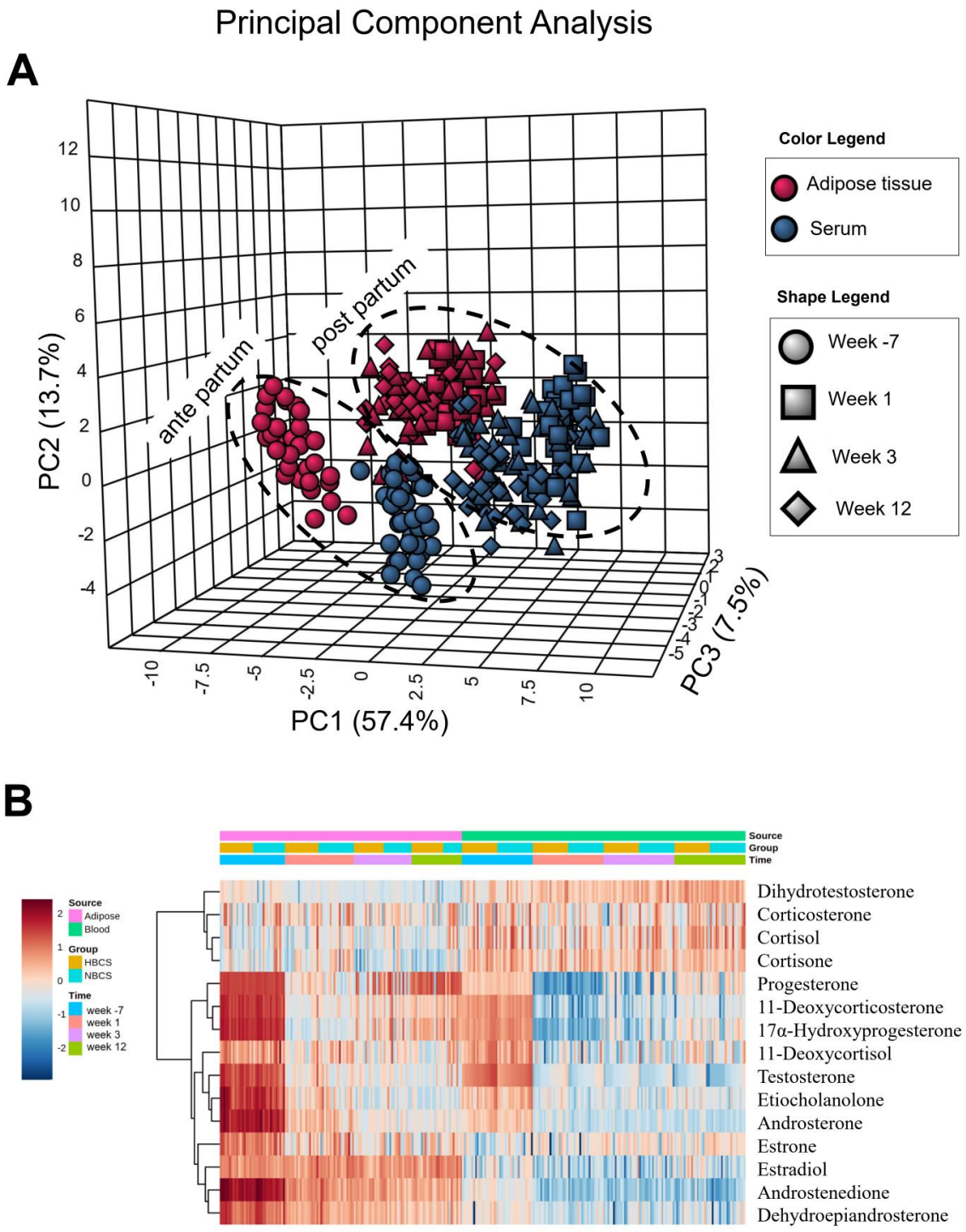


**Figure 2.** (A) Principal component analysis (PCA) showing the interaction of source (colored, adipose tissue and serum) and time of sampling (shape, weeks -7, 1, 3, 12 relative to parturition) for all log-transformed and pareto-scaled steroids regardless of body condition. (B) Clustering result for all log-transformed and pareto-scaled steroids shown as heatmap (distance measure using euclidean and clustering algorithm using ward). The color spectrum intuitively indicates the steroids abundance (mean centered and divided by the range of each variable).

**Profiles of steroid hormones in serum.** Steroid hormones measured in serum from HBCS and NBCS cows at weeks -7, 1, 3, and 12 relative to parturition are presented in Figure 3. Overall, DHEA-S was not detectable in serum. Most blood steroids changed over time with higher concentrations before than after parturition; however, the concentrations of the mineralo- and glucocorticoids aldosterone, corticosterone, cortisone, and cortisol as well as the estrogens, estrone, and estradiol, followed different patterns. Group differences were observed at week 1 p.p. for progesterone, aldosterone, and androsterone with higher concentrations (1.1-fold to 3-fold, $P < 0.05$) in HBCS than in NBCS cows. For progesterone, an interaction between group and time was observed ($P = 0.02$). Regarding parity, cows from parity class 2 (≥ 2nd and < 4th parity) had higher concentrations of progesterone, estrone (E1), dihydrotestosterone (DHT), and DHEA with nearly twice the concentrations compared to cows in parity class 3 (> 4th parity; $P < 0.05$). In contrast, cows from parity class 3 had 2.3-fold higher androstenedione concentrations compared to cows from parity class 2. Moreover, circulating mineralo- and glucocorticoids were not affected by parity.

The progesterone concentrations in serum measured by ELISA were neither affected by group nor by parity (Figure 4). The values measured by ELISA were consistently higher (around 68%) throughout the experimental period when compared to progesterone values measured by ultra-high-performance liquid chromatography-tandem mass spectrometry (LC-MS/MS). Moreover, progesterone values measured by ELISA and mass spectrometry (MS) in the 4-time points from which MS data were available, were strongly correlated ($r = 0.90$; $P < 0.001$).

**Profiles of steroid hormones in scAT.** The steroid hormones measured in scAT are presented in Figure 5. In general, the most abundant steroid in scAT was progesterone, whereas aldosterone was not detectable in scAT (aldosterone concentrations were below the limit of detection (LOD)). Group differences in scAT (observed for 11-deoxycortisol (11-DOC), corticosterone, cortisol, cortisone, androstenedione, and DHEA) were limited to the phase after parturition. For the mineralo- and glucocorticoids, concentrations were higher (1.9- to 3.5-fold, $P < 0.05$) in NBCS than in HBCS cows, whereas DHEA and androstenedione were higher (1.2- and 1.5-fold, respectively, $P < 0.005$) in HBCS compared to NBCS cows. Irrespective of time and group, cows from parity class 2 (≥ 2nd and < 4th parity) had up to 1.8-fold higher concentrations of estradiol (E2), androstenedione, and DHEA in scAT compared to cows from parity class 3 (> 4th parity).

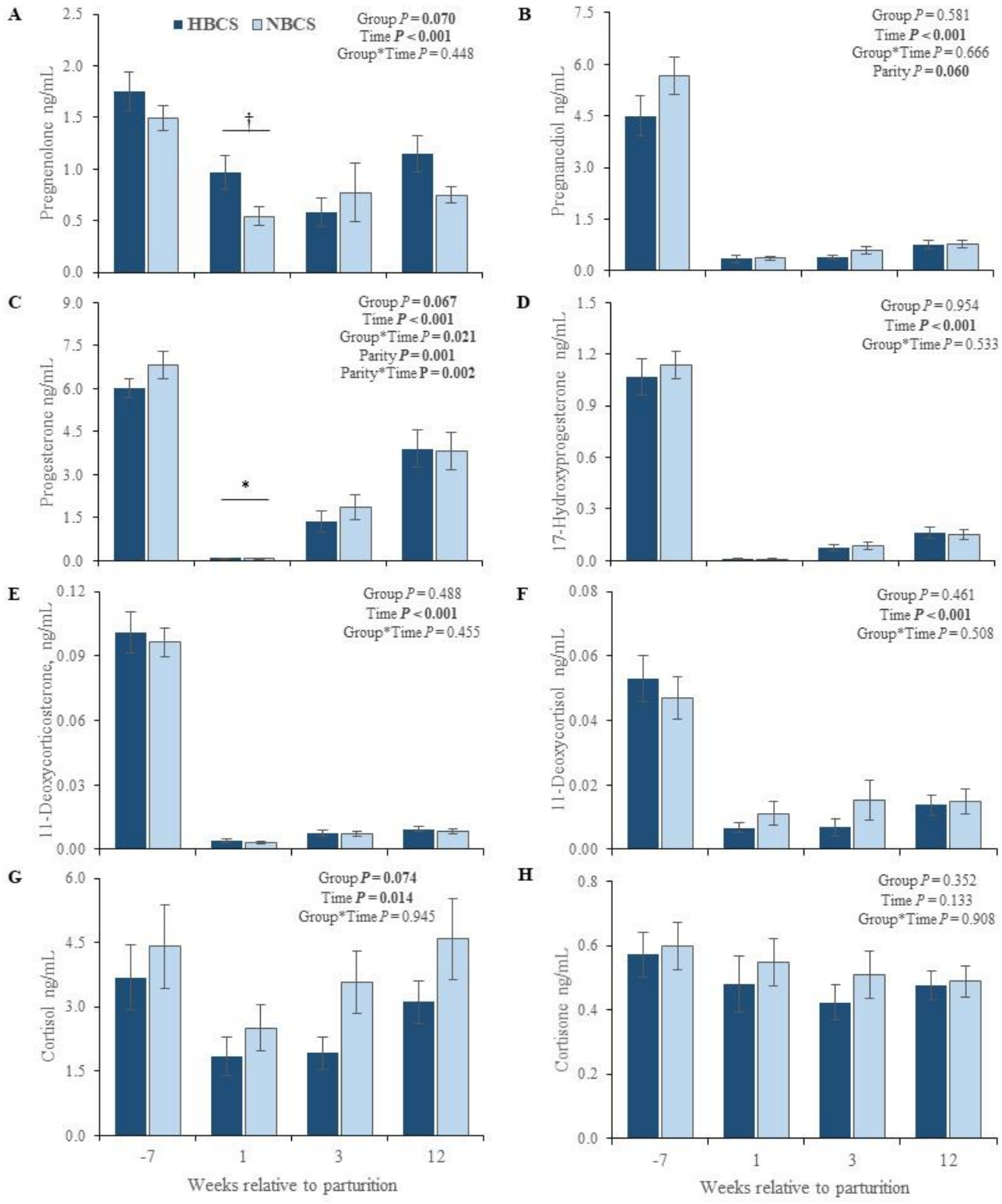


**Figure 3 (A-H).** Changes of the steroid concentrations (ng/mL) in serum of cows with normal versus high body condition score (NBCS, HBCS) at week 7 *ante partum*, as well as week 1, 3 and 12 *post partum*. Data are given as means ± SEM. Asterisks (*) indicate differences ($P \leq 0.05$) between HBCS and NBCS cows within the time points. Trends ($P \leq 0.10$) for differences between the groups are indicated by daggers (†).

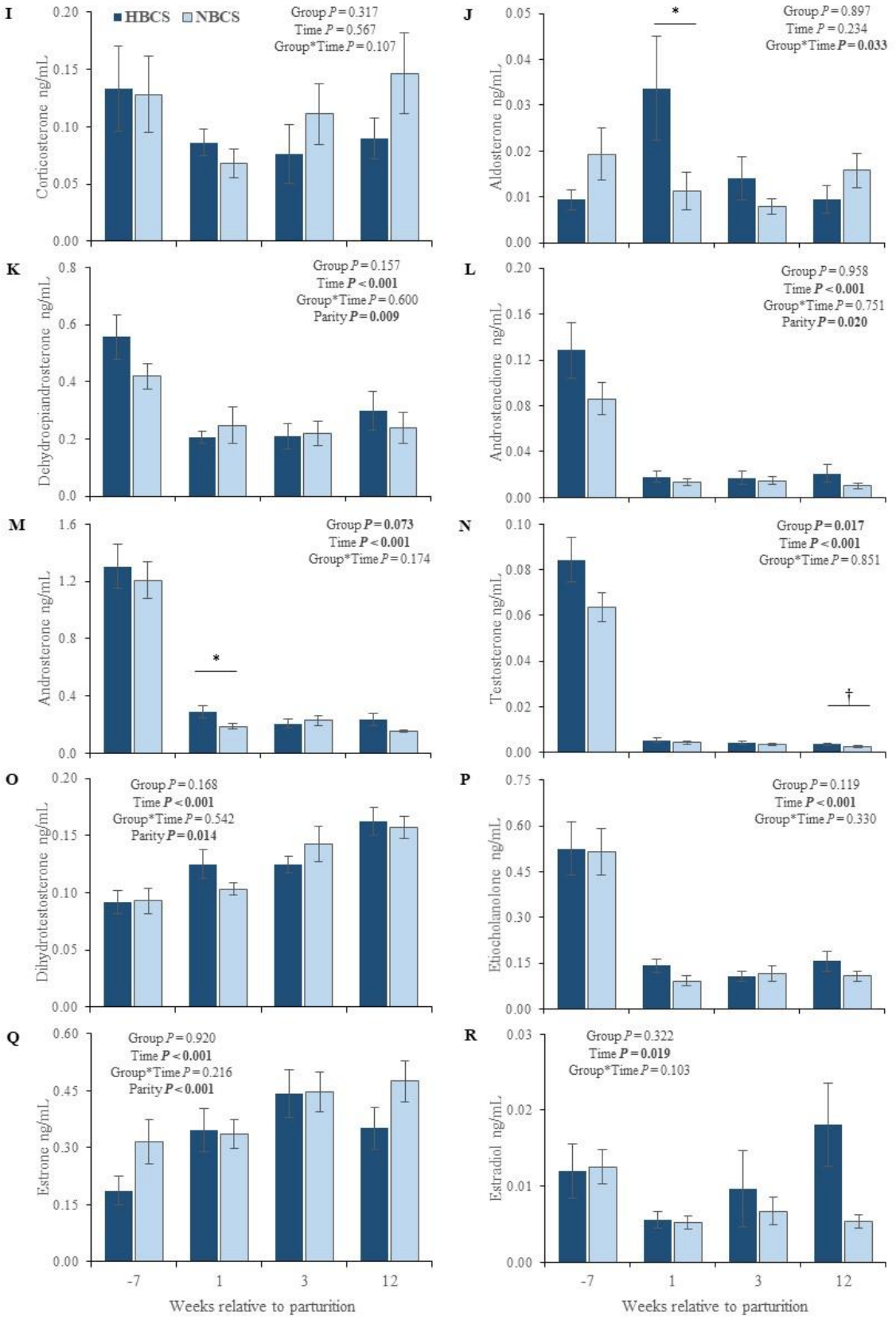


**Figure 3 (I-P).** Changes of the steroid concentrations (ng/mL) in serum of cows with normal versus high body condition score (NBCS, HBCS) at week 7 *ante partum*, as well as week 1, 3 and 12 *post partum*. Data are given as means ± SEM. Asterisks (*) indicate differences ($P \leq 0.05$) between HBCS and NBCS cows within the time points. Trends ($P \leq 0.10$) for differences between the groups are indicated by daggers (†).

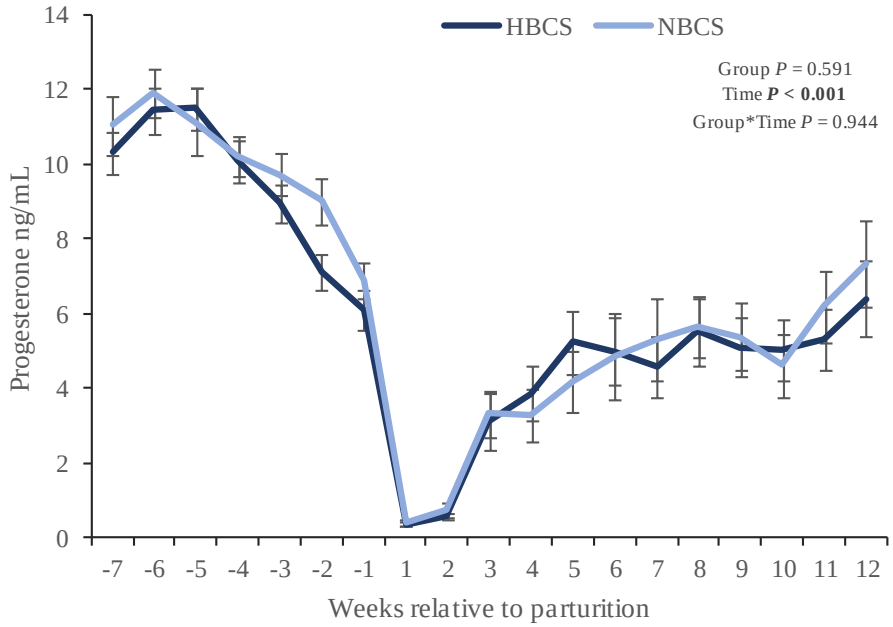


**Figure 4.** Changes in serum progesterone concentration (ng/mL) in the serum of cows with normal versus high body condition score (NBCS, HBCS) from week 7 *ante partum* to week 12 *post partum* measured by ELISA. The vertical dashed line indicates parturition. Data are given as means ± SEM. Significant value: P ≤ 0.05.

**mRNA Abundance of steroidogenic enzymes in liver and scAT.** The abundance of steroidogenic enzymes mRNA in liver and scAT are presented in Figure 6. Comparing the tissues, the mRNA abundance of *HSD3B1 (HSD3 type 1)* and *HSD11B1 (11 ß-hydroxysteroid dehydrogenase type 1)* was higher ($P < 0.001$) in liver compared to scAT at week 3 p.p.. Furthermore, the steroid *21-hydroxylase (CYP21)* mRNA abundance was higher ($P < 0.001$) in scAT than in liver at all time points (Fig. 6E & F). Before parturition, higher *HSD17B12* mRNA abundance was observed in scAT compared to liver ($P = 0.008$); however, after calving the mRNA abundance of *HSD17B12* was higher in liver ($P < 0.001$ Fig. 6G & H). Regarding group differences, the mRNA abundance of *HSD17B12* in liver was higher ($P < 0.05$) in NBCS cows compared to HBCS cows at weeks 3 and 12 p.p.. Moreover, the mRNA abundance of *HSD11B1* was higher ($P = 0.010$) at week 7 a.p. in HBCS compared to NBCS cows (Fig. 6I). In scAT, group differences were limited to *StAR* and *HSD17B12*, being higher at week 3 p.p. (*StAR*; $P = 0.035$; Fig. 6B) in NBCS compared to HBCS cows and before parturition (*HSD17B12*; $P = 0.032$; Fig. 6H) in HBCS compared to NBCS cows.

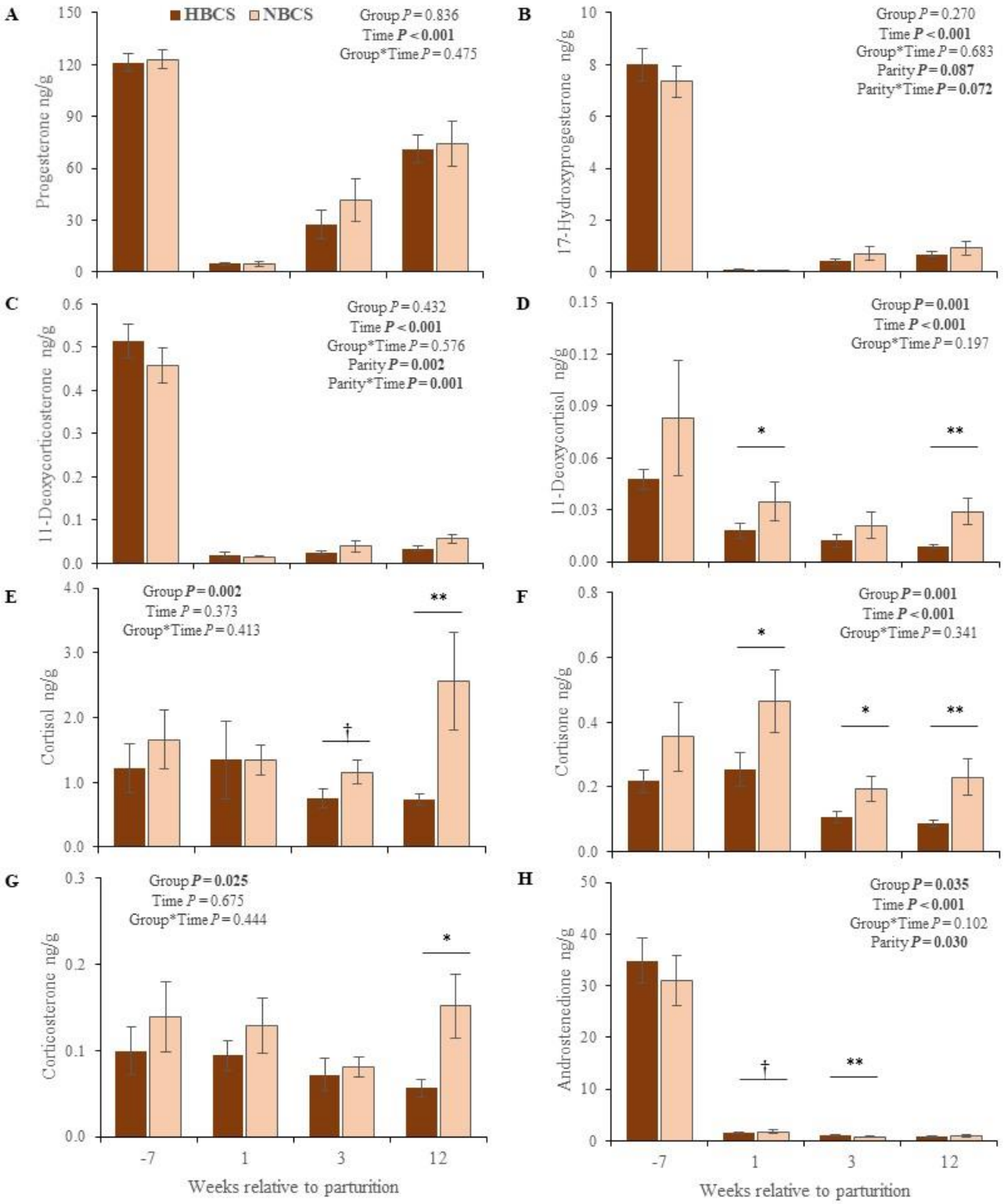


**Figure 5 (A-H).** Changes of the steroid concentrations (ng/g) in subcutaneous adipose tissue of cows with normal versus high body condition score (NBCS, HBCS) at week 7 *ante partum*, as well as week 1, 3 and 12 *post partum*. Data are given as means ± SEM. Asterisks indicate differences (*: P ≤ 0.05; **: P ≤ 0.01) between HBCS and NBCS cows within the time points. Trends (P ≤ 0.10) for differences between the groups are indicated by daggers (†).

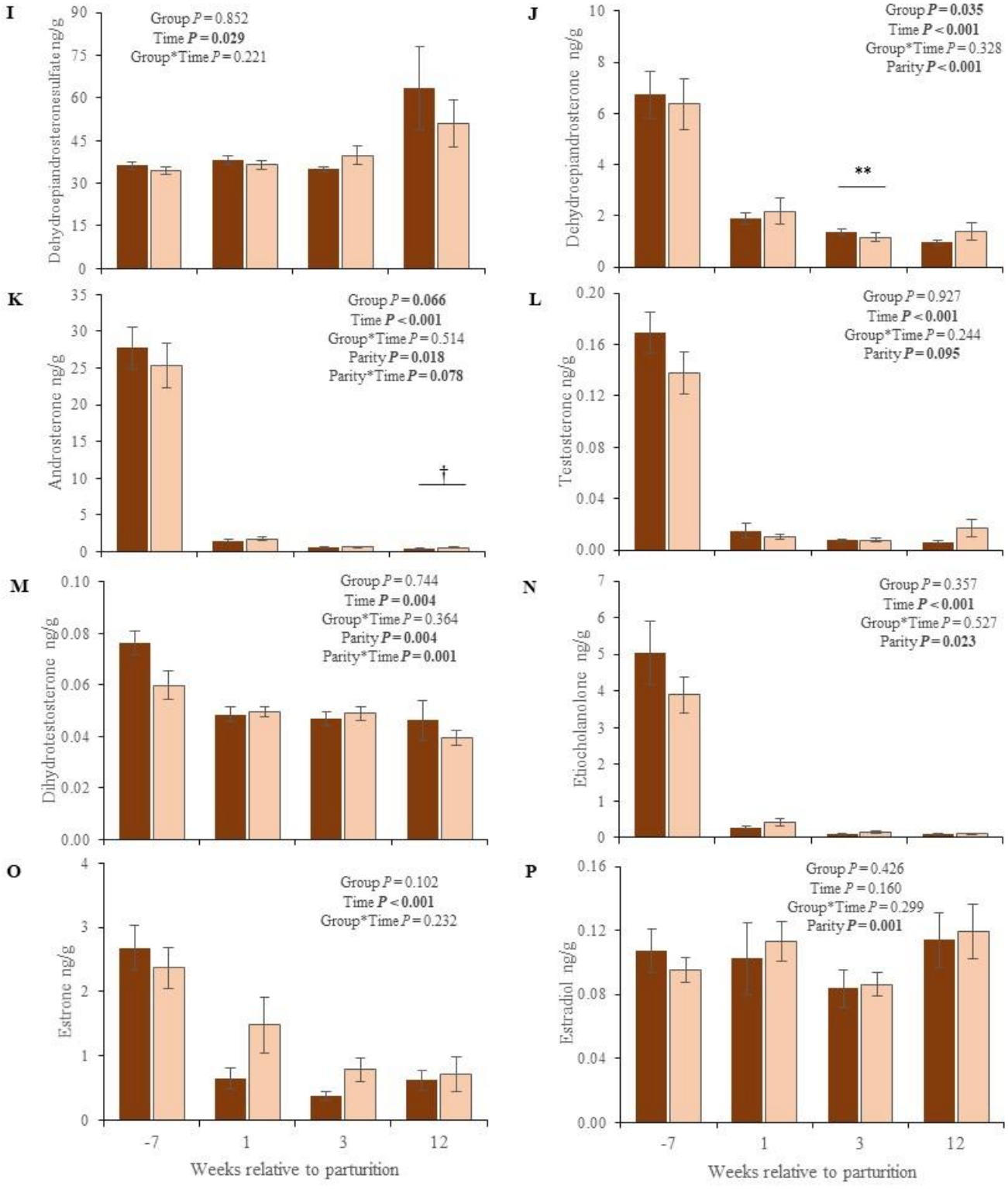


**Figure 5 (A-H).** Changes of the steroid concentrations (ng/g) in subcutaneous adipose tissue of cows with normal versus high body condition score (NBCS, HBCS) at week 7 *ante partum*, as well as week 1, 3 and 12 *post partum*. Data are given as means ± SEM. Asterisks indicate differences (*: P ≤ 0.05; **: P ≤ 0.01) between HBCS and NBCS cows within the time points. Trends (P ≤ 0.10) for differences between the groups are indicated by daggers (†).

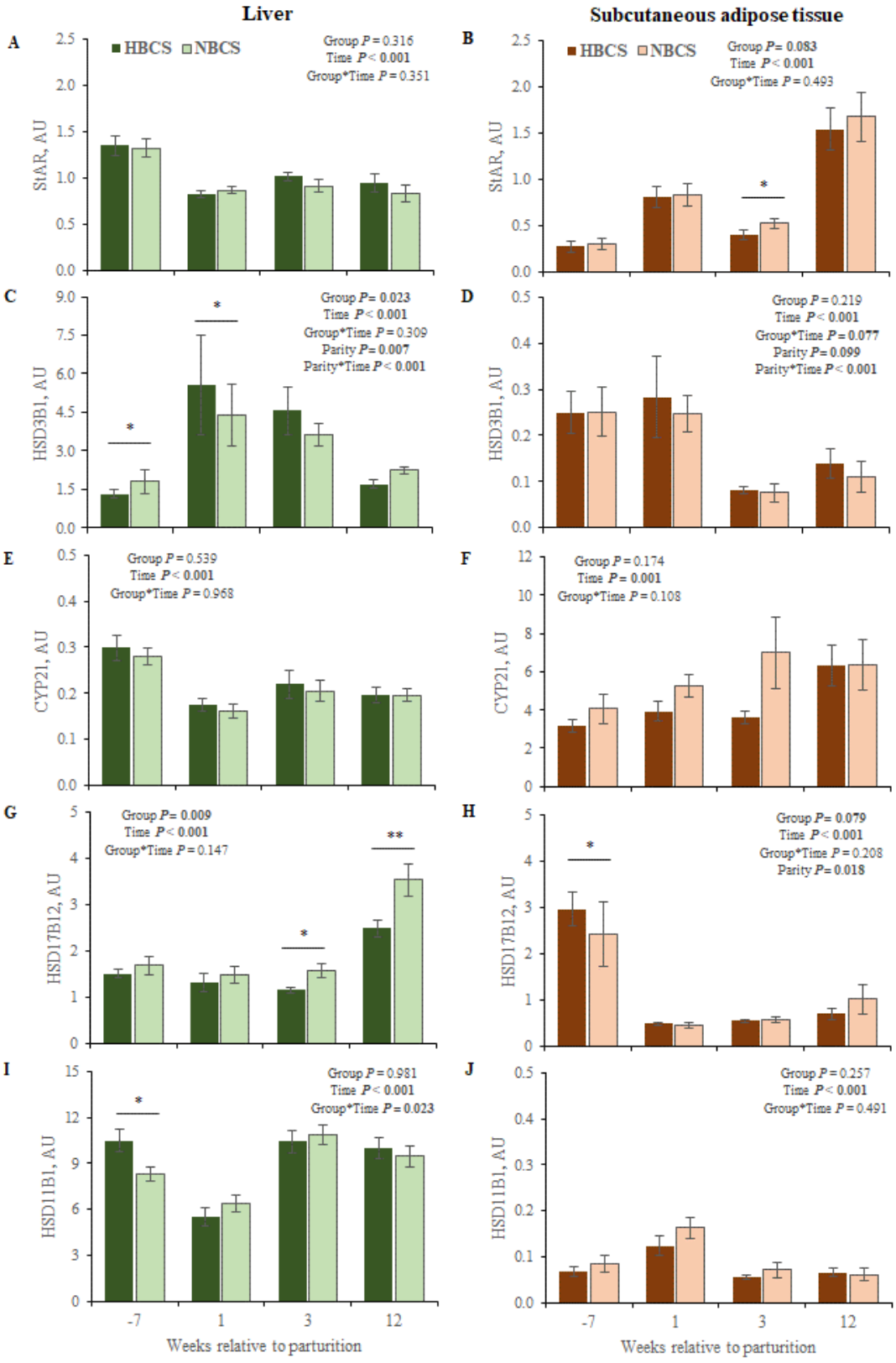


**Figure 6.** mRNA abundance of five steroidogenic enzymes [steroidogenic acute regulatory protein (*StAR*), 3 β-Hydroxysteroid dehydrogenase type 1 (*HSD3B1*), steroid 21-hydroxylase (*CYP21*), 17 ß-hydroxysteroid dehydrogenase type 12 (*HSD17B12*), and 11 ß-hydroxysteroid dehydrogenase type 1 (*HSD11B1*)], in the liver and in subcutaneous adipose tissue of cows with normal versus high body condition score (NBCS, HBCS) at wk 7 *ante partum*, as well as wk 1, 3 and 12 *post partum* (time = weeks around parturition). Asterisks indicate differences (*: P ≤ 0.05; **: P ≤ 0.01) between HBCS and NBCS cows within the time points. Trends (P ≤ 0.10) for differences between the groups are indicated by daggers (†). AU: arbitrary unit.

Parity affected *HSD3B1* mRNA abundance in liver, with higher abundance in cows from parity class 3 (> 4th parity) compared to class 2 (≥ 2nd and < 4th parity). In addition, the mRNA abundance of *HSD3B1* in the scAT was higher in cows from parity class 3 compared to cows from class 2 at weeks 7 a.p. and 12 p.p. ($P \leq 0.05$), respectively. The mRNA abundance of *HSD17B12* in scAT across all time-points was higher ($P = 0.02$) in cows with parity > 4 than in cows from class 2 (≥ 2nd and < 4th parity). For CYP21 mRNA abundance in liver, neither group nor parity of the cows affected the enzyme expression (Figure 6E). Moreover, parity did not affect *HSD11B1* mRNA abundance in both liver and scAT.

**Relationships between the steroid hormone concentrations in serum, in scAT, and the mRNA abundance of steroidogenic enzymes in scAT.** The relationships between steroid concentrations in serum and scAT as well as the associations between steroids and mRNA abundance of steroidogenic enzymes in scAT regardless of grouping and time are presented in Figure 7. Very strong correlations (r > 0.9) were observed for 11-deoxycorticosterone (11-DOCSt) and 17α-hydroxyprogesterone (17-OHP) in serum (r = 0.905; $P < 0.001$), 17-OHP in serum and 17-OHP in scAT (r = 0.916; $P < 0.001$) as well as for 17-OHP and 11-DOCSt in scAT (r = 0.931; $P < 0.001$).

**Relationships between the mRNA abundance of steroidogenic enzymes in liver and the concentration of steroids in the circulation.** Regardless of grouping and time, the mRNA abundance of enzymes *HSD11B1*, *HSD17B12*, and *StAR* in the liver were very weakly associated with blood steroids (r < 0.40; $P < 0.001$). However, the mRNA abundance of *CYP21* was moderately related to pregnanediol (r = 0.45), pregnenolone (r = 0.44), testosterone (r = 0.47), 11-DOCSt (r = 0.47), androstenedione (r = 0.46), androsterone (r = 0.40), etiocholanolone (r = 0.41), and 17-OHP (r = 0.45) (all $P < 0.001$). Furthermore, the mRNA abundance of *HSD3B* in liver was negatively associated (r < -0.40) with blood steroids, i.e. 11-DOCst, 11-DOC, 17-OHP, androstenedione, androsterone, cortisol, cortisone, DHEA, etiocholanolone, pregnanediol, pregnenolone, and testosterone; except for progesterone, a moderate negative relation was observed with hepatic *HSD3B* mRNA (r = -0.42; $P < 0.001$).

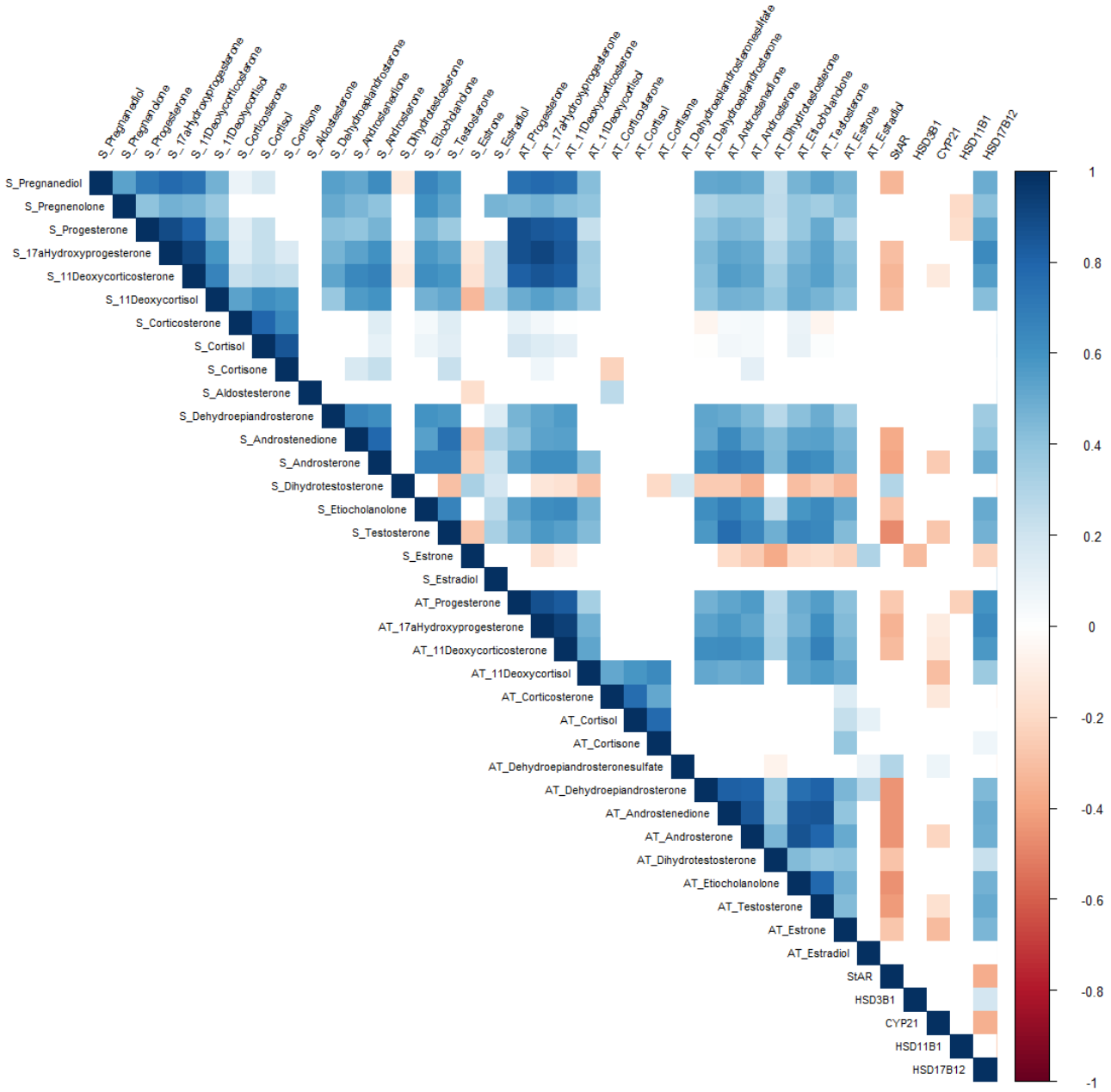


**Figure 7.** Spearman correlation plots of the steroids assessed in serum (S) and in subcutaneous adipose tissue (scAT) as well as with the mRNA abundance of steroidogenic enzymes in scAT. The correlations are based on results of overall time points and group independence. Squares are colored based on correlation coefficients (-1 to +1) with $P < 0.05$.

## Discussion

Steroid hormones are involved in various physiological processes during gestation and lactation. Due to their lipophilic character, steroids can be taken up from circulation and accumulated in AT[5,6]. In humans, steroids can be locally synthesized de novo or at least be metabolized by steroidogenic enzymes from steroid precursors[11]. With this background, we profiled the steroid concentrations in serum and scAT of periparturient dairy cows using a targeted metabolomics approach. The cows differed in their body condition before parturition, which maintained until calving; the difference was augmented by feeding a more energy-dense diet to the HBCS cows from week 15 a.p. until dry-off (7 a.p.). In particular, HBCS cows with higher BCS and BFT throughout the experimental period, lost more than twice as much BFT during early lactation when compared to NBCS cows[14]. Therefore, we hypothesized that cows with a higher body condition around parturition, releasing more fat from AT after calving, will have increased circulating steroid concentrations when compared to cows with normal body condition. Greater lipomobilization in early lactation substantiated by greater serum FA concentrations[14] might lead to an increased release of steroids from AT, and thus enhanced circulating steroid concentrations, in particular in HBCS cows.

Using gas chromatography, Bélanger *et al.* observed regional differences in human AT steroid levels, being more concentrated in omental versus scAT[17]. In addition, investigating steroid concentrations in different human AT depots (i.e. breast and abdominal AT), Szymczak *et al.* suggested local factors being involved in steroid uptake, storage, act, and metabolism[18]. Therefore, we would expect differences in steroid biosynthesis and metabolism between visceral and scAT depots in bovine. Nevertheless, besides regional differences, steroid concentrations in plasma were strongly correlated with steroid values both in omental and scAT in humans[17]. Given that biopsies from different visceral AT depots cannot routinely be sampled for tissue analyses in the alive cow, since the invasive overall sampling of AT could impair the metabolism of cows during the already challenging transition period. Therefore, we focused on scAT in the present study, albeit differences in the functional activities of visceral and scAT have been shown for dairy cows[19,20].

In the course of gestation, most steroid concentrations increase and peak near the end of term in maternal serum, whereas the steroid concentrations after parturition mostly decrease. Thus, concentrations below the LLOQ were generally found after parturition in this study. Herein, more than 50% of serum steroids measured were below the LLOQ.

Moreover, concerning 11-DOCSt, testosterone, androstenedione, and 11-DOC, 75 – 53% of the concentrations were below the LLOQ. The steroid concentrations in scAT and serum clearly differed with respect to the time before and after parturition in the current study. Furthermore, from the PCA analysis, glucocorticoids (except 11-DOC and 11-DOCst) and DHT showed distinct changes in concentration within time, source (scAT and serum), and group (NBCS and HBCS) compared to most of the sex steroids, pointing to the different function of these hormones in terms of reproduction and metabolism.

Increased circulating progesterone in cows with high versus normal body weight loss is attributed to fat mobilization after parturition and the release of progesterone stored in AT[15,16]. In the present study, greater lipomobilization in HBCS cows was accompanied by higher serum concentrations for progesterone, aldosterone, and androsterone at week 1 p.p. when compared to NBCS cows. Moreover, the trend for elevated circulating pregnenolone and the greater progesterone concentrations in the HBCS cows at week 1 p.p. might originate from increased AT mobilization in these cows. In dairy cows, the corpus luteum is the major contributor to the elevated peripheral progesterone levels during pregnancy[21]. Weekly measured progesterone concentrations in the present study followed the well-known changes of the hormone concentrations, appearing in context with parturition, i.e. progesterone decreases prior to the time of parturition and increases thereafter. Consistently higher values measured by ELISA throughout the whole experimental period compared to LC-MS/MS values, raise the problem that using different methods for quantification of the same hormone is difficult for direct comparison. However, using LC-MS/MS for multiple steroid quantification herein included also progesterone concentrations, being highly correlated to progesterone concentrations measured by ELISA.

As in humans, hyperaldosteronism was associated with metabolic syndrome, obesity, and insulin resistance[22], higher serum aldosterone concentrations at week 1 p.p. in HBCS than in NBCS cows, together with increased insulin concentrations in the periparturient period[14], might reflect reduced insulin sensitivity in HBCS cows. Comparing the present results with concentrations of FA published earlier[14], circulating aldosterone was only weakly associated with FA in week 1 p.p. ($r = 0.40$; $P = 0.03$, both groups). The relevance of androgens in female reproduction is primarily focused on their role as estrogen precursors. However, androgens per se might regulate key processes during pregnancy and parturition, e.g., androgens are believed to be critical for cervical remodeling at term and in myometrial relaxation in humans. In particular, androsterone might play a role in myometrial contractions[23].

In humans, hyperandrogenism is a key feature of polycystic ovary syndrome, with increased androgen concentrations being related to insulin resistance[24]. As mentioned for aldosterone, higher androgen concentrations, i.e. androstenedione and DHEA in scAT as well as androsterone in serum, from HBCS cows in the present study might reflect reduced insulin sensitivity in these cows. Besides interference with insulin signaling, androgens may also trigger lipolysis and thus increase FA in circulation[24]. In addition to steroids, we assessed the mRNA abundance of five key enzymes involved in steroid biosynthesis in the present study. Besides in liver, steroids can be metabolized by steroidogenic enzymes in scAT[25] and thus modulate steroid concentrations[11]. Endogenous steroids originate from their common precursor cholesterol. Together with mitochondrial outer-membrane proteins such as the translocator protein (TSPO)[26], the enzyme StAR triggers the delivery of cholesterol into the inner mitochondrial membrane (IMM), while CYP11A1 can initiate steroidogenesis through a side-chain cleavage reaction converting cholesterol to pregnenolone[6]. In mammals, pregnenolone is the main steroid synthesized from cholesterol, initiated by StAR and CYP11A[16,11] serving as a precursor for other steroids. Pregnenolone, taken up from the circulation, can be converted to 17-OHP by CYP17A1[11]. In the current study, *CYP11A1* mRNA was not detectable. Studies in murine adipocytes indicate that CYP11A1 may play only a minor metabolic role in AT[6]. However, the *StAR* mRNA was measured in scAT in our study and peaked at week 12 p.p. The higher *StAR* mRNA abundance in scAT from NBCS compared to HBCS in early lactation indicates an increased capacity of cholesterol uptake into the IMM at this time point. Cholesterol reaching the IMM can alternatively be metabolized to oxysterols instead of pregnenolone. Oxysterol 27-hydroxycholesterol is one of the major de novo adipocyte products synthesized from cholesterol by the mitochondrial enzyme CYP27A1[6]. The *de novo* synthesis of oxysterols in adipocytes was suggested to protect adipocytes against intracellular cholesterol overload and the formation of new fat cells, thus controlling the number of adipocytes upon overnutrition[6]. Increased abundance of *StAR* mRNA in scAT of NBCS cows in the present study may therefore reflect oxysterol synthesis.

Regarding the steroid concentrations in scAT, higher DHEA and androstenedione concentrations in HBCS cows p.p. may point to either an increased lipid accumulation or to a higher local metabolism of these steroids. The enzyme CYP17A1 modulates the transformation of pregnenolone to DHEA; however, in the present study, *CYP17A1* mRNA abundance was not detectable in scAT with the protocol used herein.

Considering the enzyme *HSD3B1*, increased hepatic mRNA abundance in the week after parturition was accompanied by increased androstenedione concentrations in scAT in HBCS cows p.p., thus the enzyme might contribute to peripheral conversion of androstenedione from DHEA. Both androgen precursors, DHEA and DHEA-S, were present in scAT of cows in our study, but DHEA-S was below the LLOQ in all serum samples measured herein. We cannot explain the absence of DHEA-S in bovine serum samples in this study; however, earlier studies have also observed lower DHEA-S than DHEA concentrations in circulation, suggesting a limited contribution of DHEA-S as an androgen reservoir in cows[27]. In the current study, two steroidogenic enzymes, *HSD17B12* and *HSD3B1*, involved in androgen biosynthesis were expressed in bovine scAT and liver. In humans and rodents, androgens inhibit adipogenesis and promote lipid mobilization via androgen receptors in AT[25,28]. Also, androgens can enhance the lipolytic capacity of preadipocytes by increasing the number of ß-adrenergic receptors and the activity of the enzyme adenylate cyclase[25,28]. Higher androgen concentrations in scAT from HBCS may thus prevent adipogenesis in early lactation when these cows were still mobilizing body reserves[14] (Supplemental Figure 1). Moreover, the higher mRNA abundance of *HSD3B1* in liver compared to scAT may point to hepatic synthesis of progesterone, albeit the main site of progesterone synthesis in dairy cows to maintain pregnancy is the corpus luteum[21]. Locally converted progesterone could have an anabolic role in bovine liver, since administration of progesterone increased the rate of hepatic lipogenesis in rats in vivo[29].

The interconversion of active 17-ß-hydroxy- and inactive 17-keto-steroids is catalyzed by HSD17-forms and plays an essential role in the last steps of androgen and estrogen biosynthesis. In our study, we investigated the mRNA abundance of *HSD17B12* catalyzing the synthesis of E2 from E1[9]. Moreover, *HSD17B12* is involved in the elongation process of VLCFA and is highly expressed in organs related to lipid metabolism, e.g., liver, kidney, heart, and skeletal muscle[10,30]. Depending on the concentration, *HSD17B12* could catalyze both, the elongation of FA, as well as the transformation of sex steroids[31]. Higher *HSD17B12* mRNA abundance in HBCS cows at week 7 a.p. might point to a role of the enzyme in lipid metabolism. Furthermore, higher hepatic abundance of *HSD17B12* mRNA in NBCS than HBCS cows after parturition may contribute to the same mechanism, since the NBCS cows returned earlier to a positive energy balance[14], which is likely associated with lipogenic processes. The association between *HSD17B12*, E1, and etiocholanolone, suggests a role of *HSD17B12* in converting DHT to etiocholanolone rather than E1 to E2.

Furthermore, higher E1 concentrations in scAT compared to serum may be due to greater E1 storage, increased local E1 synthesis in scAT, or a combination of both. Since E1 was increased relative to E2 in our study, rather the oxidative pathway than the reductive pathway catalyzed by HSD17 seems to be relevant. However, the gene expression of estrogenic *HSD17* enzymes in AT was reported to be lower than those of the androgenic ones; thus, androgen biosynthesis might be more relevant in AT than estrogen biosynthesis[32]. Also, the higher E1 concentrations in the scAT of our study might be due to local estrogen synthesis via steroid sulfatase enzyme (STS) and CYP19 aromatization of androstenedione[33]. However, aromatase CYP19 was not investigated in the current study. In bovine maternal circulation, E1-S is the major estrogen, indicating foetoplacental function and placental viability[34]. E1-S serves as a hormone reservoir in the circulation; in general, sulfonated steroids often exceed the concentrations of free (unconjugated) steroids in the circulation and different tissues, as also shown for DHEA-S in scAT in our study. The E1 concentrations in scAT of the cows in our study may be due to the action of STS as well as to uptake and conversion of E1-S.

Being generally known as stress hormones, glucocorticoids are mainly produced in the adrenal glands to stimulate the release of energy substrates from energy stores for use as fuel during the stress response[35]. Moreover, glucocorticoids have immune-suppressive and anti-inflammatory effects on several organs, including AT[36,37]. *In vivo* and *in vitro* studies in humans and rodents have shown that glucocorticoids also regulate lipid metabolism by stimulating lipolysis[38], diminishing preadipocyte proliferation[39], but also by encouraging adipogenesis through stimulation of differentiation from pre- to mature adipocyte[40]. In the present study, the circulating concentrations of the active glucocorticoid cortisol were within the range reported for dairy cows during the periparturient period[41]. Given that cows in a positive energy balance have no need to mobilize energy stores, the higher cortisol concentrations in scAT from NBCS cows might rather result from an accumulation of circulating cortisol, than from local synthesis. Using a proteomics analysis on plasma samples from a subset of the cows studied herein, we found that the most enriched pathways were those involved in the acute inflammatory response and regulation of humoral immune response[42]. Although glucocorticoids are not synthesized *de novo* in AT, they can be activated locally by HSD11B1[40]. In dairy cows, the enzyme activity of HSD11B1 has been measured in different AT depots[43].

Located exclusively in mature, differentiated adipocytes, an anti-inflammatory rather than a differentiating effect of cortisol was suggested in bovine AT[43]. Increased mRNA abundance of *HSD11B1* in scAT at week 1 p.p measured herein, might support the inflammatory role of cortisol in bovine AT, as demonstrated around parturition in bovine AT[44]. The liver mainly activates glucocorticoids; therefore, higher hepatic mRNA abundance of *HSD11B1* compared to scAT was not surprising. At week 7 a.p., the hepatic *HSD11B1* abundance in HBCS cows was moderately related to circulating beta-hydroxybutyrate at week 7 a.p. ($r = 0.50$; $P < 0.05$), indicating a role in lipid metabolism and ketogenesis[40]. The formation of mineralo- and glucocorticoids is triggered by the enzyme CYP21 from the precursor steroids progesterone and 17-OHP. Herein, the higher mRNA abundance of *CYP21* in scAT than in liver may suggest that mineralo- and glucocorticoids are rather converted in scAT than in liver in cattle.

In the present study, we also aimed at investigating the effect of parity on the steroid profiles in serum and in scAT as well as on the mRNA abundance of steroidogenic enzymes. Both, endocrine and metabolic parameters are affected by parity during the periparturient period in dairy cows[45]. Higher steroid concentrations in serum and in scAT from cows in parity class 2 (≥ 2nd and < 4th parity) compared to cows with parity > 4 occurred more frequently right after calving when the physiological adaptations to lactation including the mobilization of fat reserves proceed[46] and estrous cycles are resumed[8]. *In vitro* experiments revealed, that progesterone secreted from corpus luteum, as well as the mRNA expression of *StAR* and *HSD3B1*, were decreased in aged cows (mean age: 15.7 years) compared with younger cows (2.9 years) during the early luteal phase[47]. In the present study, cows with parity > 4 had more frequently higher mRNA abundances of steroidogenic enzymes in both liver and scAT compared with cows of ≥ 2nd parity and < 4th parity, suggesting an aging effect on luteal function[47] as well as on steroidogenic enzyme expression in cattle.

## Conclusion

The results of the present study indicated that not only steroid hormones but also steroidogenic enzymes in liver and scAT were altered during the periparturient period of cows.

Increased lipolysis in cows after parturition was related to higher circulating concentrations of androgens and progestagens, which might reflect the steroid release from AT into the

circulation due to fat mobilization in early lactation. Moreover, the age of the cows not only affected the steroid concentration in serum and AT, but also the mRNA abundance of steroidogenic enzymes in a local manner, depending on the time relative to parturition, which requires further investigation.

## Materials and Methods

The animal experiment was performed at the experimental station of the Educational and Research Centre for Animal Husbandry, Hofgut Neumuehle, Muenchweiler a. d. Alsenz, Germany. The experimental procedures performed in this study were in accordance with the European Union Guidelines concerning the protection of experimental animals, with approval by the local authority for animal welfare affairs (Landesuntersuchungsamt Rheinland-Pfalz, Koblenz, Germany [G 14-20-071]). The study is reported according to the ARRIVE guidelines. Cows were part of a trial aiming to establish an experimental modal for dairy cows of high versus normal body mobilization during the transition from pregnancy to lactation. Details of the experimental design were described previously[14]. In brief, 38 multiparous German Holstein dairy cows (average parity: 2.9 ± 0.3) were allocated 15 weeks before their expected calving date to either a high BCS (HBCS, n = 19) or a normal BCS (NBCS, n = 19) group. The BCS was estimated using a scale of 1 (thin) to 5 (obese) with a quarter-point system[48]; backfat thickness (BFT) was recorded in the sacral region using ultra-sonography (AGROSCAN L, ALR 500, 5MHz, linear-array transducer; Echo Control Medical, Angoulême, France). Both, BCS and BFT were continuously monitored every 2 weeks (from week 15 a.p. until week 12 p.p.). Changes of BCS and BFT from week 7 a.p. to week 13 p.p. in NBCS cows and HBCS cows were already documented[14] and are shown together with BW changes as well as their time-dependent variations in Supplemental Figure 1 and Supplemental Table 2. From week 15 until milking was stopped ("drying off") at week 7 a.p., the HBCS and NBCS cows were assigned to two feeding groups to accentuate the differences in body condition (NBCS cows: BCS < 3.5, BFT < 1.2 cm; HBCS cows: BCS > 3.75, BFT > 1.4 cm). In addition, comparable milk yields were considered for preselection of cows (NBCS: 10,361 ± 302 kg; HBCS: 10,315 ± 437 kg). The NBCS animals were fed a less energy-dense ration [6.8 $NE_L$ (MJ/kg of dry matter (DM))] than the HBCS animals [7.2 $NE_L$ (MJ/kg of DM)] from week 15 a.p. until week 7 a.p.. At dry off, the groups had the targeted difference in BCS and BFT: HBCS with 3.8 ± 0.1 (min/max range: 3.0 to 4.5; IQR: 0.7) and 2.0 ± 0.1 cm (min/max range: 1.3 to 2.9 cm; IQR: 0.8), respectively, and NBCS with 3.0 ± 0.1 (min/max

range: 2.75 to 3.25; IQR: 0.3) and 0.9 ± 0.1 cm (min/max range: 0.4 to 1.4 cm; IQR: 0.4), respectively. During the subsequent dry period, and the lactation thereafter, both groups received the same diets. The cows obtained all diets for ad libitum intake as TMR consisting of 74% roughage and 26% concentrate in the low-energy ration and 63% roughage and 3% concentrate in the high-energy ration. A detailed description of the ingredients as well as the composition of the diets is given in Supplemental Table S1. The calculations of energy contents of the diets were performed according to the recommendations for lactating cows of the German Society of Nutrition Physiology[49]. The differences in body condition were largely maintained until calving, during lactation BCS declined in both groups, with greater BCS losses in HBCS cows than in NBCS sows (see Supplemental Figure 1 and Supplemental Table 2).

**Sampling**

Blood samples for steroid metabolomic analyses were collected after the morning milking but before feeding, on weeks -7, 1, 3, and 12 relative to parturition from the *V. caudalis mediana* as previously described[14]. Serum was obtained from blood after clotting and subsequent centrifugation (10 min, 2,000 x g at 4°C) and stored at -80°C until analysis. Progesterone was measured in serum samples obtained weekly from weeks -7 until +12 relative to parturition using an in-house developed ELISA[50]; the inter- and intra-assay coefficient of variations were 7.9% and 6.7%, respectively. Moreover, biopsies from liver as well as scAT from the tailhead region were collected from both groups on weeks -7, 1, 3, and 12 relative to parturition as described recently[51]. In brief, tissue biopsies were collected under local anesthesia (Procaine hydrochloride, 20 mg/mL, Richter Pharma AG, Wels, Austria) and while the cows were sedated (xylazine i.v., 20 mg/mL, 0.1 mL/100 kg of BW; CP-Pharma Handels GmbH, Burgdorf, Germany) and fixed in a headlock. Liver biopsies were obtained by liver puncture at the 11$^{th}$ and 12$^{th}$ intercostal space using a 14-gauge biopsy needle (Dispomed Witt oHG, Gelnhausen, Germany). For the scAT biopsies, a 1 cm skin incision was made in the tailhead region and scAT from the underlying fat layer was collected. All tissue samples were rinsed with sterile 0.9% NaCl solution, immediately snap-frozen in liquid nitrogen, and stored at -80 °C until analysis.

**Quantification of steroid hormones in serum samples.** The following 19 steroids were quantified in serum (250 µL) using an extended version of the Absolute*IDQ*™ Stero17 assay (Biocrates Life Sciences AG, Innsbruck, Austria) and LC-ESI-MS/MS[52]: aldosterone, androstenedione, androsterone, corticosterone, cortisol, cortisone, 11-DOCSt, 11-DOC, DHEA, DHEA-S, DHT, E1, E2, etiocholanolone, 17-OHP, progesterone, testosterone, pregnenolone, and pregnanediol (the last two steroids were assessed semi-quantitatively). Mass spectrometric analyses were performed on a QTRAP 5500 triple quadrupole system (Sciex Deutschland GmbH, Darmstadt, Germany) equipped with a 1260 Series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) and a HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) controlled by the software Analyst 1.6.2. Compound identification and quantification were based on scheduled multiple reaction monitoring measurements (sMRM). Sample preparation and LC-MS/MS measurements were performed as described by the manufacturer´s protocol (UM-STERO17, Biocrates AG). Samples were handled using a Hamilton Microlab STAR™ robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) and a Waters Positive Pressure-96 Processor (Waters GmbH, Eschborn, Germany). Until analysis, all samples were stored at -80 °C. A detailed method description has been published[53].

In brief, ultrapure water (400 µL), internal standard mix (20 µL), blank (250 µL), calibration standards, quality control samples, or serum samples were mixed in a 96 deep well plate. The SPE (solid phase extraction) plate of the kit was conditioned successively with dichloromethane (1 mL), followed by acetonitrile (1 mL), methanol (1 mL), and ultrapure water (1 mL). After plate conditioning, the mixed samples were loaded onto the SPE plate, and steroids were subsequently eluted in two steps: 1) twice with 500 µL dichlormethane into the same deep well plate and 2) with 600 µL acetonitrile into another deep well plate. After drying with nitrogen, the dichloromethane fraction was dissolved in 50 µL methanol/water (25/75 v/v), whereas the acetonitrile fraction was diluted with 400 µL water. Both plates were centrifuged at 50 x g and placed into the cooled auto sampler (10 °C) for LC-MS/MS measurements. The LC-separation of both fractions was performed using 470 mL ultrapure water and the content of three ampules of the kit as mobile phase A and acetonitrile/methanol/ultrapure water v/v/v 85/10/5 as mobile phase B (initial 35%, rising up to 100%). After sample injection (20 µL), steroids were separated at a flow rate of 300 µL and at 45°C column temperature on the HPLC column for the Absolute*IDQ*™ Stero17 Kit combined with the precolumn SecurityGuard Cartridge C18 4 x 2 mm (for HPLC,

Phenomenex Cat No. AJ0-4286; Phenomenex, Aschaffenburg, Germany). The method of the Absolute*IDQ*™ Stero17 Kit were in conformance with the EMEA-Guideline[54], which implies proof of reproducibility within a given error range. Analytical specifications for LOD, LLOQ and ULOQ (lower and upper limit of quantification), specificity, linearity, precision, accuracy, reproducibility, and stability were determined experimentally by Biocrates and are described in the user manual AS-STERO17-3.

Data evaluation for quantifying the steroid concentrations and quality assessment was performed with the software MultiQuant 3.0.1 (Sciex) and the Met*IDQ*™ software package, which is an integral part of the Absolute*IDQ*™ Stero17 Kit. Metabolite concentrations were calculated using internal standards and reported in nM or ng/mL. Due to unspecific contaminations, pregnanediol and pregnenolone could not be detected in 6 out of 38 samples at week 7 a.p. [2 NBCS, 4 HBCS], and 4 out of 38 samples after calving at week 1 [1 NBCS, 3 HBCS], week 3 [2 NBCS, 2 HBCS], and week 12 [4 HBCS].

**Quantification of steroid hormones in scAT.** In bovine scAT, the same steroid hormones (except pregnenolone, pregnanediol, and aldosterone) as already described for serum were determined by LC-MS/MS based on the Absolute*IDQ*™ Stero17 assay (Biocrates Life Science AG). The QTRAP 5500 triple quadrupole system (Sciex) was equipped with a Turbo V ion spray interface and coupled to an Agilent 1290 Infinity UHPLC-system (two G7120A binary pumps, a G7116B column thermostat), and a G7167B multi-sampler (Agilent Technologies). Instrument control and data acquisition were performed with Analyst Software Version 1.7, and for data evaluation MultiQuant Version 3.0.3 was used (Sciex). Sample processing for steroid analysis in scAT was divided in two main steps. The first step was an in-house method (Helmholtz Zentrum München). The extracts obtained during this procedure were used to perform the second step of sample processing using the Absolute*IDQ*™ Stero17 assay (Biocrates Life Science AG). For steroid extraction, scAT (100 mg), internal standard solution (10 µL), methanol (240 µL), and pure water (250 µL) were homogenized (Precellys® 24, Bertin instruments, Montigny-le-Bretonneux, France). After homogenization, methanol (1 mL) was added, vortexed and incubated for 10 min at 50 °C and 800 rpm (Thermomix comfort, Eppendorf, Hamburg, Germany). Afterwards, tubes were centrifuged at 15,000 x g and 4 °C, for 15 min (Mikro 200R Hettich centrifuge, Bäch, Switzerland) and subsequently stored at -20 °C for 30 min. The supernatant was transferred to a glass vial and evaporated to dryness (Barkey vapotherm, Barkey GmbH, Leopoldshöhe, Germany) under nitrogen at

30 °C. Finally, the residue was dissolved in methanol/water (250 µL; 5/95), and transferred to a 96 well plate provided with the Absolute*IDQ*™ Stero17 assay (Biocrates Life Science AG). In addition, blanks (250 µL), calibration solutions, and quality control samples were pipetted to the well plate. Finally, the steroids were subsequently eluted from the SPE plate. The first extract was obtained by elution with dichloromethane. After evaporation of the eluent, the residue was dissolved in methanol/water (50 µL; 25/75 vol/vol). This extract contained the steroids except for DHEA-S. A second extract was obtained by eluting the SPE-plate with acetonitrile. The eluate was diluted with water to get a final volume of 600 µL (acetonitrile/water 66/34 vol/vol) containing DHEA-S. Both extracts were subjected to LC-MS/MS analysis.

The liquid chromatographic separation of the steroids was performed on a core-shell column (Kinetex RP-18, 1.8 µm, 150 x 2.1 mm I.D. with a Security Guard Ultra Cartridge 2.1 mm I.D) from Phenomenex. During separation, the column was operated at 45 °C at a flow rate of 0.3 mL/min applying elution with binary gradients from 65 % A to 0 % A (A: formic acid (0.1 %)/2-propanol, 97/3, v/v; B: acetonitrile/methanol/water, 85/10/15, v/v/v). The injection volume was 20 µl for the dichloromethane (steroids except for DHEA-S) and 10 µL for the acetonitrile extract (DHEA-S). Tandem mass spectrometric detection and quantification of the steroids were done by electrospray ionization and sMRM.

Quality parameters were assessed to demonstrate the performance of the analytical method for the determination of steroids in scAT. Regardless of the internal standards, recoveries of the steroids were between 19.9% and 101% with CVs from 6.9% to 39.6% (n = 10). Considering the internal standards, recoveries were between 48.9% and 111% with CVs from 5.5% to 19.0%. The calculation of recovery and repeatability based on the respective internal standards significantly improved the reliability and was thus recognized as indispensable; moreover, the use of substance-specific, individual internal standards is necessary. The LODs and LOQs were calculated based on signal-to-noise ratios 3:1 and 10:1, respectively. The LODs were between 0.002 ng/g and 0.3 ng/g scAT, LOQs ranged from 0.007 ng/g to 0.9 ng/g scAT. Imprecisions for within-day-analysis (n = 7) - as a measure of repeatability - ranged from 6.8 % to 15.5 % (CVs). The calibration functions were linear with regression coefficients > 0.995.

**Extraction of RNA and Quantitative Real-Time PCR in scAT and liver samples.** Details of the RNA extraction and cDNA synthesis were described previously[51]. After tissue homogenization with the Precellys 24 system (VWR/Peqlab Biotechnologie, Erlangen, Germany) total RNA was extracted from liver and scAT by using the TRIzol reagent (Invitrogen/Life Technologies, Carlsbad, CA, USA) according to the manufacturer´s protocol. The RNA was purified with spin columns according to the Qiagen kit protocol (RNeasy Mini Kit, Qiagen GmbH, Hilden, Germany). The concentration of total RNA and the purity was quantified at 260 nm and 280 nm using the Nanodrop 1000 (peQLab Biotechnology GmbH, Erlangen, Germany). For cDNA synthesis, a reverse transcription of 250 ng total RNA per 20 µL reaction volume was performed with RevertAid reverse transcriptase (Thermo Scientific GmbH, Dreieich, Germany) according to the manufacturer's instructions with a Multicycler PTC 200 (MJ Research Inc, Watertown, MA, USA). Quantitative real-time PCR (qPCR) was carried out using an MX3000p PCR cycler (Stratagene, Amsterdam, the Netherlands, and Agilent, Santa Clara, CA, USA) in accordance with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines[55]. The qPCR conditions and primer sequences used in the present study are presented in Table 1. Samples were run as triplicates in a total volume of 10 µL, with 2 µL of cDNA (diluted 1:4) as a template, 1 µL of assay-specific primer mix, 2 µL of water, and 5 µL of DyNAmo ColorFlash SYBR Green qPCR Kit (Thermo Fisher Scientific, Dreieich, Germany). Each run included a negative-template control for quantitative PCR, as well as a negative-template control and no-reverse-transcriptase control of cDNA. Relative quantification of the target genes, i.e. *HSD11B1, HSD3B1, HSD17B12, StAR,* and *CYP21* was performed with standard curves using cDNA serial dilutions to calculate the abundance based on run-specific PCR efficiency. For each PCR, a set of two inter-run calibrators was used to correct for inter-run variation.

Target genes were normalized based on the most stable reference genes in liver and scAT, i.e. low-density lipoprotein receptor-related protein 10 (*LRP10*), RNA polymerase II (*POL2*), eukaryotic translation initiation factor 3 subunit K (*EIF3K*), hippocalcin-like protein 1 (*HPCAL1*), and emerin (*EMD*), determined by geNorm$^{PLUS}$ algorithms of qBASE$^{plus}$ 3.1 software (Biogazelle, Ghent, Belgium).

**Table 1.** Primer characteristics of target and reference genes used in adipose tissue and liver and the real-time polymerase chain reaction conditions.

| **Target Genes**[1] | Sequences (5'-3') | NCBI Accession No. | Fragment size (bp[3]) | Annealing (s/◦C)[4] | Extension (s)[5] |
|---|---|---|---|---|---|
| *HSD17B12* | | | | | |
| Forward | GCTGCTAAAACCCTGACCCA | NM_001101307.1 | 101 | 60/59 | 60 |
| Reverse | GAGTGGCCTGGTGTCATTCA | | | | |
| *CYP21* | | | | | |
| Forward | CGTGAAGGGCACTGAGAAAT | NM_174639.1 | 100 | 60/59 | 60 |
| Reverse | AGGTGGGAGCTGAACGTCTA | | | | |
| *StAR* | | | | | |
| Forward | GTGGATTTTGCCAATCACCT | NM_174189.2 | 202 | 30/62 | 15 |
| Reverse | TTATTGAAAACGTGCCACCA | | | | |
| *HSD3B1* | | | | | |
| Forward | TGTTGGTGGAGGAGAAGGATCTG | NM_174343 | 207 | 30/59 | 15 |
| Reverse | GCATTCCTGACGTCAATGACAGAG | | | | |
| *HSD11B1* | | | | | |
| Forward | AAGCAGACCAACGGGAGCATT | AF548027 | 112 | 60/60 | 60 |
| Reverse | GGAGAAGAACCCATCCAGAGCA | | | | |
| **Reference Genes** [2] | | | | | |
| *LRP10* | | | | | |
| Forward | CCAGAGGATGAGGACGATGT | BC149232 | 139 | 30/61 | 20 |
| Reverse | ATAGGGTTGCTGTCCCTGTG | | | | |
| *POL2* | | | | | |
| Forward | GAAGGGGGAGAGACAAACTG | X63564 | 86 | 60/60 | 30 |
| Reverse | GGGAGGAAGAAGAAAAAGGG | | | | |
| *HPCAL1* | | | | | |
| Forward | CCATCGACTTCAGGGAGTTC | NM_001098964 | 99 | 30/60 | 30 |
| Reverse | CGTCGAGGTCATACATGCTG | | | | |
| *EIF3K* | | | | | |
| Forward | CCAGGCCCACCAAGAAGAA | NM_001034489 | 125 | 45/59 | 30 |
| Reverse | TTATACCTTCCAGGAGGTCCATGT | | | | |
| *EMD* | | | | | |
| Forward | GCCCTCAGCTTCACTCTCAGA | NM_203361 | 100 | 45/59 | 30 |
| Reverse | GAGGCGTTCCCGATCCTT | | | | |

[1] *HSD17B12 = 17 ß-hydroxysteroid dehydrogenase type 12; CYP21 = steroid 21-hydroxylase; StAR = steroidogenic acute regulatory protein; HSD3B1 = 3 β-Hydroxysteroid dehydrogenase type 1; HSD11B1 = 11 ß-hydroxysteroid dehydrogenase type 1*

[2] *LRP10 = lipoprotein receptor-related protein 10; POL2 = RNA polymerase II; HPCAL1 = hippocalcin-like protein 1; EIF3K = eukaryotic translation initiation factor 3 subunit K; EMD = Emerin.*

[3] Base pairs

[4] Initial denaturation for 10 min at 90°C; denaturation for 30 s at 95°C; 40 cycles, except for *StAR*, *HSD3B1*, and *HPCAL1* (35 cycles), *LRP10* (33 cycles).

[5] Extension at 72°C.

## Statistical Analysis

Statistical analyses for steroid hormones and steroidogenic enzymes were carried out by using a linear mixed model with repeated measurements (IBM SPSS Version 21). Before statistical analysis, the normality of data distribution was tested using the Shapiro-Wilk test using the UNIVARIATE procedure and evaluated visually by plotting residuals.

All dependent data (steroids, steroidogenic enzymes mRNA) were not normally distributed; therefore, data were log (base 10) transformed to meet the assumptions of normality and homoscedasticity of the residuals. The data presented in this paper show the non-transformed values of the data (mean ± SEM); however, all P-values were calculated using the transformed data. The mixed models used herein contained the fixed effects of treatment (group: HBCS and NBCS), time (weeks relative to parturition), and the interaction between treatment and time, while the individual "cow" was considered as a random factor. The effect of BCS antepartum was included in the model as a fixed effect of the group (treatment). Moreover, parity (defined in classes) and the interaction with time was considered as a fixed effect; however, when insignificant it was excluded from the model. Multiparous cows were assigned to parity classes regarding their number of lactations at the beginning of the experimental period (i.e. BCS grouping): class 1: = 1$^{st}$ parity cows entering 2$^{nd}$ parity (n = 6), class 2: ≥ 2$^{nd}$ and < 4$^{th}$ parity (n = 14); class 3: ≥ 4$^{th}$ parity (n = 18). The Bonferroni correction was used for multiple comparisons. Differences with $P \leq 0.05$ were considered significant and a trend was defined at $0.05 > P \leq 0.10$.

Relationships between variables (non-transformed data) were tested by Spearman correlation using R[56]. Associations were tested for the periods before and after parturition, for the entire experimental period, as well as for each group separately. Correlation figures for the whole period were computed using the R package corrplot[57]. Correlations were shown based on their significance level of $P < 0.05$, whereas non-significant correlations were not illustrated ("blank"). Moreover, the web-based metabolomics data processing tool MetaboAnalyst 4.0 was used for principal component analysis (PCA) and heat map (distance measure using euclidean and clustering algorithm using ward) of the steroids data[58]. Before data analysis, a data integrity check was performed to make sure that all the necessary information had been collected. Variables containing more than 0.6% missing values (i.e. values lower than LOD) were not considered for steroid PCA. The metabolite data were transformed using the generalized log transformation and then Pareto-scaled (mean-centred and divided by the square root of the standard deviation of each variable) to correct for heteroscedasticity, to reduce the skewness of the data, and to reduce mask effects[59].

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## Acknowledgment

The authors like to thank all co-workers at the Educational and Research Centre for Animal Husbandry, Hofgut Neumühle who supported the animal trial. Special gratitude is dedicated to Inga Hofs and Isabella Israel (Institute of Animal Science, Physiology and Hygiene Unit, University of Bonn, Germany) for her excellent laboratory assistance. K. Schuh was a recipient of a scholarship from the H. Wilhelm Schaumann Foundation. We thank Dr. Guanghua Wang, for performing the progesterone ELISA analysis during his internship at the University of Bonn. Moreover, we thank Maria Kugler, Julia Scarpa, and Werner Römisch-Margl for metabolomics measurements performed at the Helmholtz Zentrum München, Genome Analysis Center, Metabolomics Core Facility.

This study was partly supported by a grant from the German Federal Ministry of Education and Research (BMBF) to the German Center Diabetes Research (DZD e.V.) as well as by the German Research Foundation (DFG, HA 6026/4-1).

## Author contributions

S.H., H.Sau. and H.Sad. designed and supervised the entire study. K.S., D.F., C.K. and G.D. planned and performed the farm trial. M.H.G. did further data analysis. K.S. and S.H. wrote the original draft. C.P., J.L. analysed the steroid metabolomics. All authors revised the manuscript.

## Funding

This study was partly supported by a grant from the German Federal Ministry of Education and Research (BMBF) to the German Center Diabetes Research (DZD e.V.) as well as by the German Research Foundation (DFG, HA 6026/4-1).

## Additional Information

**Supplementary information:** The data that support the findings of this study are available from the corresponding author, [S.Häussler], upon reasonable request.

**Competing Interests:** The authors have no conflicts of interest to declare.

**Supplemental Table 1.** Ingredients and chemical composition of the diets (from Schuh et al., 2019)

| | Late lactation | | Dry period | Early lactation |
|---|---|---|---|---|
| | Wk 15 to 7 a.p. | | Wk 7 a.p. to parturition | Wk 1 to 14 p.p. |
| Item | HBCS | NBCS | HBCS / NBCS | HBCS / NBCS |
| **Ingredient** | | | | |
| Grass silage | 22.4 | 32.0 | 32.0 | 22.4 |
| Corn silage | 20.7 | 32.0 | 32.0 | 20.7 |
| Pressed beet pulp silage | 12.5 | - | - | 12.5 |
| Hay | 5.5 | 5.4 | 5.4 | 5.5 |
| Straw | 2.3 | 4.1 | 4.1 | 2.3 |
| Vitamin and mineral mix[1] | 0.4 | 0.7 | 0.7 | 0.4 |
| Concentrate[2] | 36.2 | 25.8 | 25.8 | 36.2 |
| **Chemical composition** | | | | |
| ME (MJ/kg DM) | 10.8 | 10.6 | 10.6 | 10.8 |
| $NE_L$ (MJ/kg DM) | 7.2 | 6.8 | 6.8 | 7.2 |
| Crude protein (g/kg DM) | 170 | 157 | 157 | 170 |
| Utilizable crude protein (g/kg DM) | 156 | 149 | 149 | 156 |
| $aNDF_{OM}$ † (g/kg DM) | 359 | 382 | 382 | 359 |
| $ADF_{OM}$ ‡ (g/kg DM) | 204 | 223 | 223 | 204 |
| Ruminal N balance (g/d) | 3.4 | 2.3 | 2.3 | 3.4 |

[1]Provided per kg total mixed ration (on DM basis): Ca, 0.36 g; P, 0.36 g; sodium, 0.36 g; Mg, 0.40 g; Zn, 28 mg; Mn, 17 mg; Cu, 6.0 mg; Co, 0.24 mg; I, 0.80 mg; Se, 0.21 mg; vitamin A, 4.000 IU, vitamin D, 600 IU, vitamin E, 20 mg (RINDAMIN K11 ATG, Schaumann, Pinneberg, Germany).

[2]Concentrate portion consisting of barley (6.5% of DM), corn grain (8.8% of DM), soybean meal (5.9% of DM), and canola meal (6.5% of DM)

† $aNDF_{OM}$, ash free neutral detergent fiber of organic matter

‡ $ADF_{OM}$, acid detergent fiber of organic matter

**Supplemental Table 2:** Body condition score (BCS), back fat thickness (BFT), and body weight (BW) loss of animals with high (HBCS) versus normal (NBCS) body condition score before calving

| **Time** | | **wk -1 until +3** | **wk -1 until +7** | **wk -1 until +11** | **wk -1 until +15** |
|---|---|---|---|---|---|
| **Group** | **Parameter** | [mean ± SEM; (%)] | [mean ± SEM; (%)] | [mean ± SEM; (%)] | [mean ± SEM; (%)] |
| **HBCS** | BCS loss | 0.62 ± 0.06 (15 %) | 0.97 ± 0.06 (23 %) | 1.08 ± 0.06 (26%) | 1.17 ± 0.09 (28 %) |
| | BFT loss, cm | 0.52 ± 0.07 (21 %) | 1.05 ± 0.10 (43 %) | 1.28 ± 0.11 (53%) | 1.37 ± 0.14 (64 %) |
| | BW loss, kg | 140 ± 7 (17 %) | 171 ± 13 (20 %) | 177 ± 13 (21 %) | 215 ± 13 (25 %) |
| **NBCS** | BCS loss | 0.42 ± 0.07 (12 %) | 0.63 ± 0.06 (18 %) | 0.68 ± 0.08 (19%) | 0.82 ± 0.11 (23 %) |
| | BFT loss, cm | 0.30 ± 0.06 (23 %) | 0.51 ± 0.06 (38 %) | 0.66 ± 0.06 (49%) | 0.69 ± 0.08 (51%) |
| | BW loss, kg | 102 ± 7 (14 %) | 109 ± 8 (15 %) | 112 ± 8 (15 %) | 121 ± 10 (16 %) |
| *P* value† | BCS | 0.036 | < 0.001 | < 0.001 | 0.020 |
| | BFT | < 0.001 | < 0.001 | < 0.001 | < 0.001 |
| | BW | 0.001 | < 0.001 | < 0.001 | < 0.001 |

†*P*-value based on UNIANOVA (GLM)

**Supplemental Table 3.** Number of cows and number of cases affected by clinical conditions occurring from calving to the first 6 weeks after calving in HBCS and NBCS cows (from Webb et al., 2020).

| | HBCS (n = 19) | | NBCS (n = 19) | |
|---|---|---|---|---|
| Clinical condition | No. cows | No. cases | No. cows | No. cases |
| Mastitis | 8 | 16 | 6 | 11 |
| Ketosis | 4 | 5 | 2 | 2 |
| Milk fever | 4 | 5 | 2 | 4 |
| Locomotion | 7 | 8 | 3 | 4 |
| Retained fetal membranes/endometritis | 1 | 1 | 2 | 2 |
| Other conditions[1] | 3 | 4 | 3 | 3 |
| Total clinical conditions | 27 | 39 | 15 | 26 |

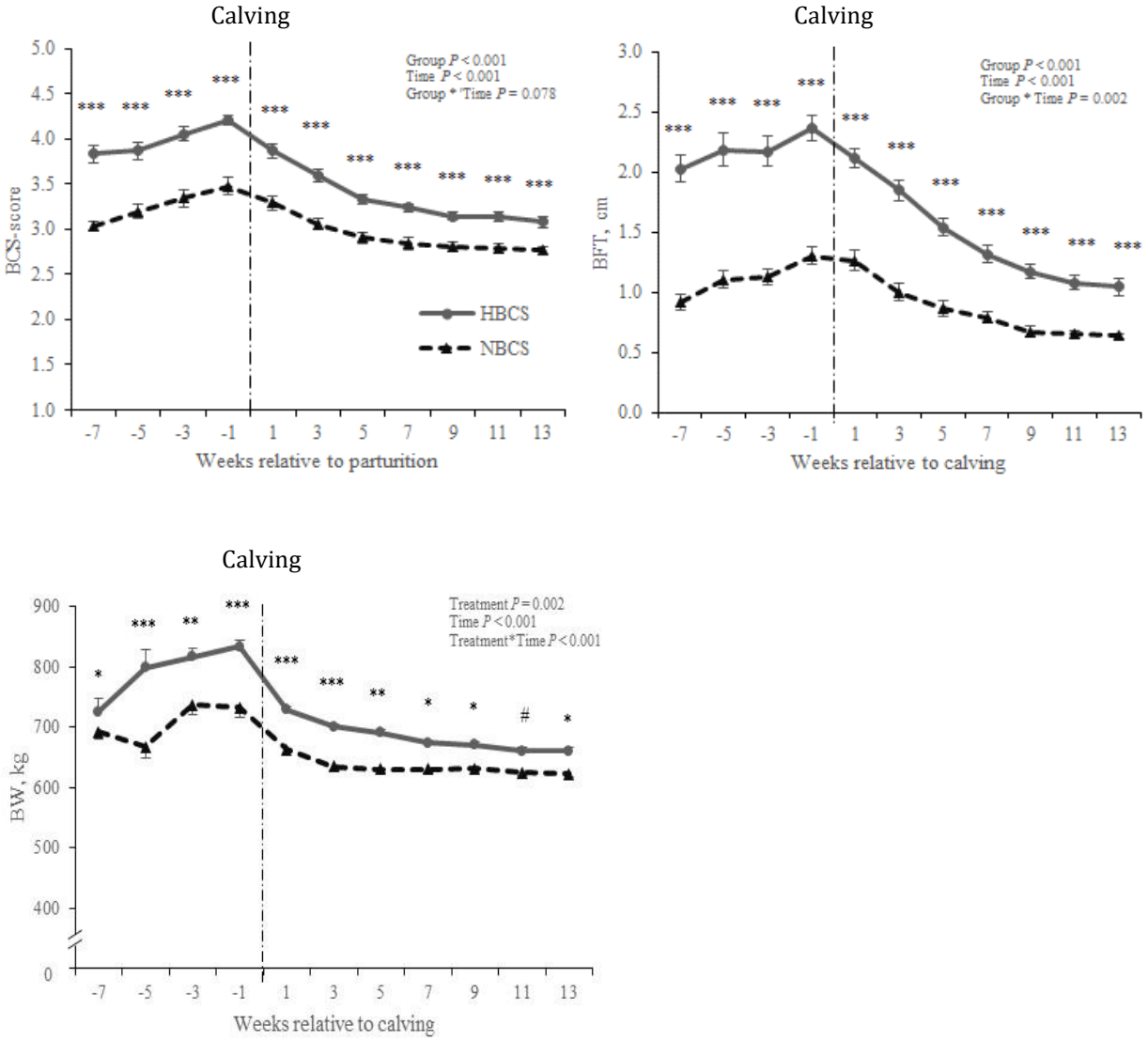
[1] dystocia, inflammation of the conjunctiva of the eye

**Supplemental Table 4.** The steroids determined by the LC-MS/MS methods, their transitions, and the internal standards used for quantification. The first transition was used for quantification, the second one (if available) for qualification. Steroids are listed in the order of their elution from the analytical columns.

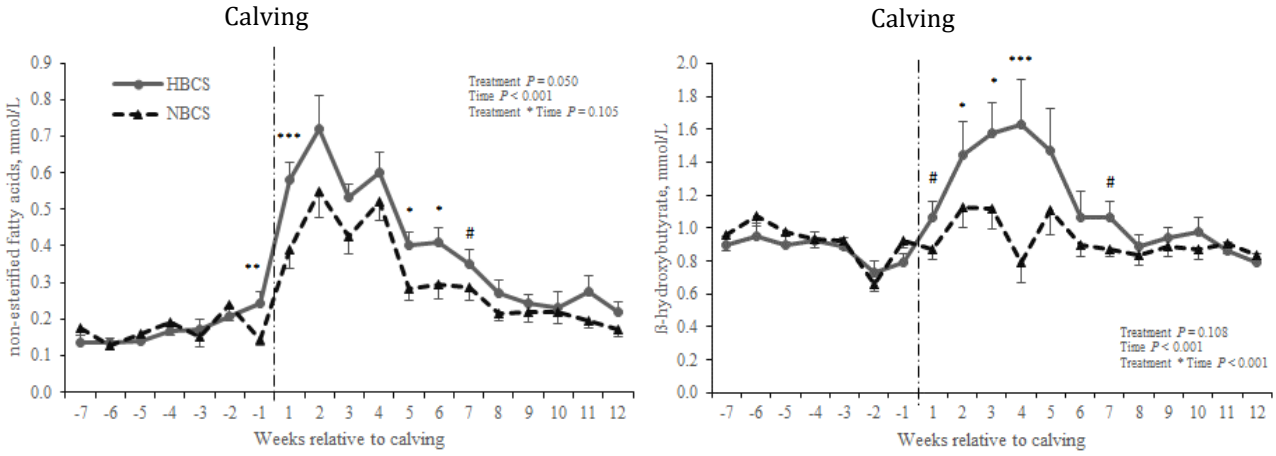
| Steroid | Transitions (m/z) | Internal standard |
|---|---|---|
| Dehydroepiandrosterone sulfate [a] | 367.2 → 97.0<br>367.2 → 80.1 | d6- Dehydroepiandrosterone sulfate |
| Aldosterone [b] | 361.1 → 343.2<br>361.1 → 315.2 | d7-Aldosterone |
| Cortisol | 363.1 → 345.1<br>363.1 → 121.1 | d4-Cortisol |
| Cortisone | 361.1 → 163.1<br>361.1 → 105.0 | d7-Cortisone |
| Corticosterone | 347.1 → 329.2<br>347.1 → 121.1 | d8-Corticosterone |
| 11-Deoxycortisol | 347.1 → 97.1<br>347.1 → 109.1 | d5-11-Deoxycortisol |
| Estradiol | 255.1 → 159.1<br>255.1 → 133.2 | d3-Estradiol |
| Testosterone | 289.1 → 97.0<br>289.1 → 109.1 | d5-Testosterone |
| 11-Deoxycorticosterone | 331.1 → 97.1<br>331.1 → 109.1 | d8-11-Deoxycorticosterone |
| Estrone | 271.1 → 133.1<br>271.1 → 253.2 | d4-Estrone |
| Androstenedione | 287.1 → 97.1<br>287.1 →109.1 | d3-Androstenedione |
| Dehydroepiandrosterone | 271.1 → 253.2<br>271.1 → 133.1 | d4- Dehydroepiandrosterone |
| 17-Hydroxyprogesterone | 331.1 → 97.1<br>331.1 → 109.1 | d8-17-Hydroxyprogesterone |
| Dihydrotestosterone | 291.1 → 255.2 | d3-Diyhydrotestosterone |
| Etiocholanolone | 273.1 → 255.2<br>291.2 → 255.2 | d4-Etiocholanolone |
| Androsterone | 273.1 → 255.2<br>291.2 → 255.2 | d4-Androsterone |
| Progesterone | 315.1 → 109.1<br>315.1 → 97.1 | d9-Progesterone |

[a]: For negative ion mode (unscheduled MRM) the dwell time was 25 ms.

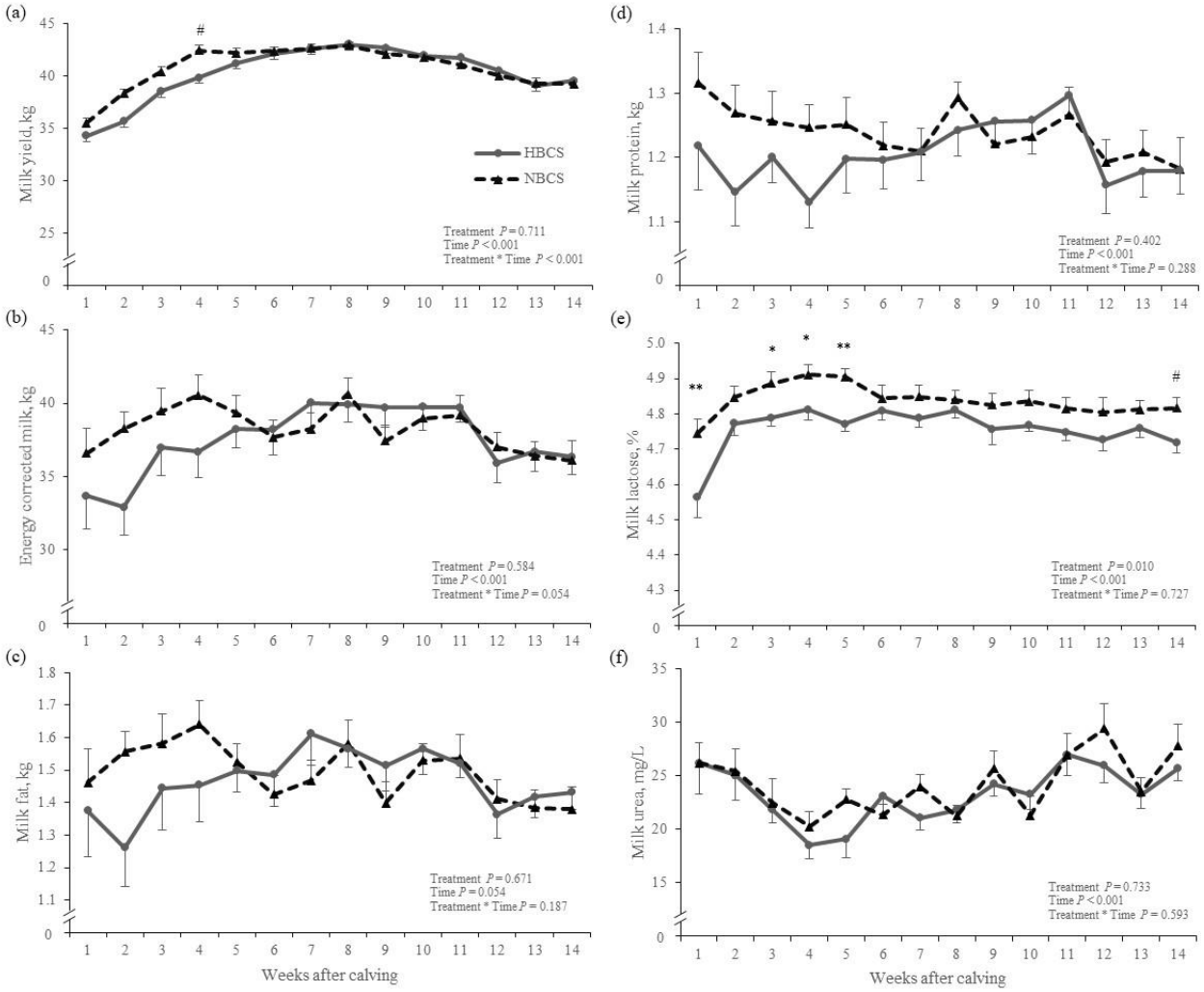
[b]: For positive ion mode (scheduled MRM) the target scan time per sMRM experiment was 0.5 s. Aldosterone was not determined in scAT.




**Supplemental Figure 1.** Changes in body condition score (BCS), backfat thickness (BFT), and body weight (BW) from week 7 ante partum to week 13 postpartum of cows with normal versus high body condition score (NBCS, HBCS; each n = 19). Data are given as means ± SEM. Asterisks indicate differences (*: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; #: $0.05 > P \leq 0.10$) between HBCS and NBCS within one time point. The vertical dashed line indicates parturition. The vertical dashed line indicates parturition. Data were already published by Schuh et al. (2019).




**Supplemental Figure 2.** Changes in non-esterified fatty acids (nmol/L) and ß-hydroxybutyrate (nmol/L) from week 7 ante partum to week 13 postpartum of cows with normal versus high body condition score (NBCS, HBCS; each n = 19). Data are given as means ± SEM. Asterisks indicate differences (*: $P \leq 0.05$; **: $P \leq 0.01$; ***: $P \leq 0.001$; #: $0.05 > P \leq 0.10$) between HBCS and NBCS within one time point. The vertical dashed line indicates parturition. Data were already published by Schuh et al. (2019).




**Supplemental Figure 3.** Yields of (a) milk, (b) energy-corrected milk, (c) milk fat and (d) milk protein, and concentrations of (e) lactose and (f) urea in milk in high body condition score (HBCS) or normal body condition score (NBCS) cows from 1 to 14 weeks *postpartum* (time = weeks relative to calving). Results are presented as means ± SEM. Significant differences between the groups are indicated with asterisks (*) when P ≤ 0.05 or (**) when P ≤ 0.01 at a given time point, respectively. Trends (P ≤ 0.10) for differences between the groups at a given time point are indicated by (#). Data were already published by Schuh et al. (2019).

# 5 General discussion

The objectives of the current study were to investigate the extent of lipid mobilization in cows with high (HBCS) versus normal body condition scores (NBCS) prior to calving and to compare changes of steroid hormone concentration in serum and scAT during the transition from late pregnancy until peak lactation between the two groups. We further examined the mRNA expression of several key enzymes involved in steroid biosynthesis, in the liver and scAT of HBCS and NBCS cows. It was hypothesized that the augmented mobilization of adipose tissue after calving in over-conditioned cows contributes to changes in circulating and AT steroid concentrations along with differences in the expression of steroidogenic enzymes compared with normal conditioned cows. Further, the expression of steroidogenic enzymes in liver and scAT of the cows was also assumed to be subjected to longitudinal changes around parturition.

## ***5.1*** *Periparturient changes in performance and metabolism in overconditioned versus normal conditioned dairy cows*

For the animal model used herein, cows were pre-selected according to the body condition of their current and previous lactation 15 weeks prior to parturition. To further push the differences in body condition, NBCS cows were fed a low-energy ration [6.8 NEL (MJ/kg of DM)], while HBCS cows were fed the higher-energy fresh cow ration [7.2 NEL (MJ/kg of DM)] from week 15 to 7 before the expected calving date. Thereafter, both groups received the same rations during the dry period and subsequent lactation. The HBCS group had higher values in BCS and BFT already at allocation 15 weeks before calving compared to the NBCS group. Differential feeding further augmented these differences during late gestation, which remained stable until the end of the observation period (12 weeks *post partum*). These findings underpin that pre-selecting animals earlier according to their spontaneously developed BCS and amplifying body condition by intense feeding until dry-off is more effective in inducing over-condition in cows than dietary intervention only during the dry period or parts of it. This suggestion was based on studies in which such pre-selection was done (Smith et al. 1997; Schulz et al. 2014) and differences in body condition and also the expected changes in metabolism were successfully induced. In contrast, in studies without such preselection, but feeding diets with different energy content during the dry period and also thereafter, no effects on BCS at calving were observed (Roche et al. 2006; Roche et al. 2009), and the concentrations of metabolic parameters after calving were hardly changed

(Tienken et al. 2015). Consequently, for comparing the metabolic adaptations in over-conditioned and in normal conditioned cows, experimental designs incorporating the predisposition to higher body fat accumulation appear to be more reliable than differential feeding alone and support the notion that a genetic predisposition, in addition to dietary intervention, contributes to the extent of lipogenesis and lipolysis during the periparturient period in cows (Rocco and McNamara 2013).

In recent decades, an optimal dry period (DP) length has been established, ranging from 40 to 60 days, resulting in little or no loss of milk yield in the following lactation (Bachman and Schairer 2003). In the herd management within our study, cows were dried off approximately 49 days prior to the expected calving date. However, the DP length was longer on average in HBCS than NBCS cows before the start of the trial (mean ± SD: 70±25 and 45±12 days, respectively; $P < 0.01$) which was further influenced by parity ($P < 0.05$). It has been previously shown that multiparous cows have prolonged DP lengths compared to primiparous cows (Atashi et al. 2013), while in our study cows ≥ 4 parities showed increased DP length compared to cows with ≤ 3 parities. The increased DP length without milk production in HBCS cows may also have contributed to the elevated body condition, as also observed in overconditioned cows in other studies (Weber et al. 2015). Assuming that the extended DP length resulted in an increase in body condition in HBCS cows, there were still cows in both groups with extended DP (> 60 days) that did not exhibit extreme weight gain during this period. Similarly, Chen *et al.* (2016) demonstrated that cows with a 60-d DP had lower BCS at the onset of lactation than cows with a 0-d or 0→67-d DP, suggesting that not only the DP length is determinant for fattening cows. An extended DP length often occurs due to prolonged calving interval (CI) or insufficient milk production. Therefore, cows with prolonged CI may have more time to replenish their fat depots and increase their body condition (Weber et al. 2015). The CI of cows in our study was numerically longer in HBCS than NBCS cows (398±63 and 375±39 days, respectively; $P = 0.166$).

In addition, an extended voluntary waiting period (WP; date of calving to the date of first insemination), extended lactation period, or service period (SP; date of calving to the date of successful conception) may also promote body condition increase in cows. Niozas *et al.* (2019) observed that cows with extended voluntary WP of 180 days exhibited higher BCS and BFT at dry off when compared to cows with a voluntary WP of 40 and 120 days. The WP in HBCS and NBCS cows in our study was 79±29 and 72±16 days ($P = 0.328$), respectively, which is within the range of voluntary WP (30 to 90 days; DeJarnette et al. 2007) reported

for dairy cows. Accordingly, lactation period – or days of milking – did not differ between the HBCS and NBCS group (325±64 and 331±39 days, respectively; $P$ = 0.735). The SP was numerically higher in HBCS than NBCS cows before trial start (120±64 and 96±38 days, respectively; $P$ = 0.163), indicating an increased number of artificial inseminations per pregnancy (AI). Nevertheless, the number of AI did not differ between the groups (HBCS: 2.3±1.5, NBCS: 1.9±1.0; $P$ = 0.376), which may be explained by the limited number of animals enrolled in this study and the high variation within groups. The cows in our study were selected for their predisposition to higher body condition in the previous lactation, which was further promoted by differential feeding prior to dry off (see explanation above) and likely resulted in the targeted differences between groups. However, due to the limited number of animals in this study it remains uncertain whether other determinants (such as extended DP length, service period, etc.) may also contribute to animal overconditioning.

The increased body condition in the HBCS group resulted in a more pronounced decrease in BCS and BFT after parturition compared to NBCS cows. This was also accompanied by lower feed intake in HBCS versus NBCS cows during the first few weeks after calving, which is known to be more pronounced in cows with elevated BCS prior to calving (Roche et al. 2009). The decline in DMI in HBCS cows resulted in a more severe NEB along with increasing serum NEFA and BHB concentrations, confirming the increased lipolysis in HBCS versus NBCS cows. In addition, hyperketonemia (BHB > 1.2 mml/L) occurred more frequently in HBCS cows (HBCS cows: 83% versus NBCS cows: 61%) and lasted longer than in NBCS cows. According to the hepatic oxidation theory (Allen et al. 2009), increased hepatic fatty acid oxidation due to increased NEFA load in HBCS cows probably caused a satiety signal in these cows, leading to decreased DMI already 3 weeks before parturition. A decrease in voluntary feed intake is often observed in dairy cows close to term, accompanied by elevated NEFA and BHB levels due to the onset of lipolysis (Bell 1995; Petterson et al. 1994). The decrease in DMI before parturition was also attributed to decreasing progesterone and increasing estrogen concentrations inducing anorexia (Muir et al. 1972; Grummer 1993), confirming the influence of steroids on lipid homeostasis (Tchernof et al. 2015). However, the regulation of periparturient DMI involves many factors (Grummer et al. 2004) and is poorly understood (Drackley et al. 2005).

Recently published results derived from the experimental approach described herein also indicated inadequate metabolic adaptations in HBCS compared with NBCS cows due to increased circulating concentrations of acylcarnitines (Ghaffari et al. 2020a) and long-chain

acylcarnitines (Ghaffari et al. 2019a) in over-conditioned cows, possibly causing mitochondrial overload and impaired fatty acid ß-oxidation in early lactation.

During periods when the nutrient intake cannot meet the increasing energy requirements of the mammary gland, body reserves are mobilized, mainly from the AT to compensate for the lack of energy supply (Bell 1995). Regardless of whether a cow is in an appropriate nutritional condition prior to parturition, every lactating cow typically experiences some type of negative energy balance, where up to 1 kg of AT can be mobilized each day (Vernon 1998). With reference to the estimates of Schröder and Staufenbiel (2006), who proposed that a change in BFT of 1 mm corresponds to app. 5 kg of total body fat (TBF), HBCS cows in our study mobilized about 65 kg of TBF until week 12 of lactation, whereas NBCS cows mobilized about 34 kg TBF. Although this may be a very simplified calculation, HBCS lost almost twice as much body fat (also demonstrated by changes in BCS and BFT) compared to NBCS cows. In addition, the elevated concentrations of circulating NEFA and BHB in HBCS versus NBCS cows indicated a more intense lipid mobilization in over-conditioned cows. Although mobilization of body reserves was more extensive in HBCS compared with NBCS cows, production efficiency was not considerably reduced in HBCS cows: except that milk yield tended to be lower in HBCS than in NBCS cows two weeks p.p., both groups showed comparable 100-day milk performance (HBCS: 3,816 ± 114 kg; NBCS: 3,875 ± 93 kg). Higher circulating NEFA and BHB in HBCS cows may have served as an additional source of energy for milk fat synthesis by the mammary glands (Herdt 2000; Drackley et al. 2001) and compensated the deficiency of dietary energy intake of HBCS cows in early lactation.

Milk composition differed slightly between groups, although NBCS cows showed higher milk-fat (kg) and milk-protein (kg) compared to HBCS cows during the first 4 weeks of lactation. Thereafter, the differences disappeared. Accordingly, the energy corrected milk (ECM) tended to be 16 and 11% higher in weeks 2 and 4 in NBCS compared to HBCS cows. In addition, milk-lactose concentrations were up to 0.18 percentage points higher in NBCS compared to HBCS cows until 5 weeks after calving. The uptake of glucose by mammary glands can be independent of arterial concentrations (Nielsen et al. 2001), as indicated by the higher circulating glucose but lower milk lactose concentrations in HBCS relative to NBCS cows in our study. Even though HBCS cows consumed less feed during the first weeks after calving, they were able to maintain milk yield at a similar level to NBCS cow, likely as a result of greater lipid mobilization compared to NBCS cows.

Leptin is involved in the control of energy homeostasis and food intake and its blood concentrations are positively correlated with BCS, BW, and adipocyte size in dairy cows (Locher et al. 2015). In the current study, circulating leptin was higher in HBCS than NBCS cows during pregnancy and early lactation and was moderately to highly correlated with body condition parameters during the periparturient period, in agreement with the results of other studies (Kokkonen et al. 2005). In addition, leptin concentration decreased in the overconditioned cows from 3 weeks a.p. onward along with decreasing DMI, indicating leptin resistance during late gestation and early lactation (Ehrhardt et al. 2016). Similarly, the periparturient insulin and glucose concentrations were higher in HBCS than in NBCS cows, suggesting decreased insulin sensitivity (IS) during the periparturient period.

Concomitant investigations from this experimental setup examined circulating microRNA (miRNA) profiles in HBCS and NBCS cows in combination with pathway enrichment analysis at different periparturient time points (Webb et al. 2020a). During the periparturient period and independent of grouping, the most enriched pathways of differentially expressed miRNAs were associated with cell cycle and insulin signaling, as well as glucose and lipid metabolism (Webb et al. 2020a). These pathways were down regulated right after parturition (Webb et al. 2020a), supporting the well-known notion of ruminants being insulin resistant in early lactation (Bell 1995).

The gold standard for measuring insulin resistance in dairy cows has been established by direct measurement of insulin sensitivity using the Hyper-insulinemic Euglycemic Clamp (HEC) test (DeFronzo et al. 1979; de Koster and Opsomer 2013). However, this measurement is a time- and labor-intensive method that requires extensive animal experimentation. The revised quantitative insulin sensitivity check index (RQUICKI = 1/[ log glucose (mg/dL) + log insulin (µU/mL)+ log NEFA (mmol/L)]) is used as a simplified and recognized surrogate marker for measuring IS (Perseghin et al. 2001; Holtenius and Holtenius 2007). Low RQUICKI values indicate decreased IS which has been detected in prepartum overconditioned dairy cows after parturition (de Koster et al. 2015). In our study, HBCS cows also showed approximately 30% lower RQUICKI in the first week after calving relative to NBCS cow ($P < 0.05$). In addition, when BHB was included in the equation to form $RQUICKI_{BHB}$ (= 1/[ log glucose (mg/dL) + log insulin (µU/mL)+ log NEFA (mmol/L) + log BHB (mmol/L)]; Balogh et al. 2008), HBCS had approximately 20% lower $RQUICKI_{BHB}$ values at week 1 ($P < 0.05$) and 4 p.p. ($P < 0.01$) compared to NBCS cows.

The lower values of the surrogate indices (RQUICKI and RQUICKI$_{BHB}$) are attributable to the increased NEFA and BHB values in HBCS versus NBCS cows at the beginning of lactation. These results provide further evidence that HBCS cows were far more metabolically challenged compared to NBCS cows, along with decreased IS in the first few weeks in milk.

Other recent publications derived from this animal experiment described herein have also demonstrated reduced muscle phosphatidylcholine and lysophosphatidylcholine levels in HBCS relative to NBCS cows in peak lactation, possibly corroborating anomalies in insulin signaling and inflammation in the muscle of overconditioned cows (Sadri et al. 2020). In addition, a quantitative proteomics approach in combination with gene ontology enrichment analysis to investigate changes in the plasma proteome of normal and overconditioned cows using a subset of 5 cows from each group representing the greater or lower extremes within each group described herein (high HBCS subset: HE-HBCS with 4.50 < BCS > 3.75, fatty acids (FA) = 1.17 ± 0.46 mmol/L, and BHB = 2.15 ± 0.42 mmol/L (means ± SD) and low NBCS subset: LE-NBCS with 3.50 < BCS > 2.75, FA = 0.51 ± 0.28 mmol/L, and BHB = 0.84 ± 0.17 mmol/L; Ghaffari et al. 2020b) revealed 24 differently abundant proteins in the LE-NBCS versus HE-HBCS cows. Here, the most enriched terms in the LE-NBCS compared to HE-HBCS group were proteins involved in the acute inflammatory response, regulation of complement activation, protein activation cascade, and regulation of humoral immune response (Ghaffari et al. 2020b). These results are in line with the findings of the first manuscript of this thesis: HBCS cows showed impaired oxidative capacity due to lower plasma ferric reducing ability (FRAP) along with higher oxidative stability index values (OSi: dROM/FRAP; unpublished data) compared to NBCS cows. Lower antioxidant protection and elevated production of free radicals, indicated by reactive oxygen metabolites, are likely to be present in dairy cows with pronounced BCS losses after calving (Bernabucci et al. 2005).

Interestingly, the companion studies also revealed that in spite of extreme mobilization of body reserves, HBCS cows showed increased mRNA expression of key components within the mammalian target of rapamycin pathway (mTOR) and the ubiquitin-proteasome system (UPS) in skeletal muscle during early lactation compared with NBCS cows (Ghaffari et al. 2019c). The increasing mRNA abundance of the aforementioned components in HBCS cows may reflect immediate activation of catabolic and anabolic processes and higher protein turnover to avoid extreme losses of skeletal muscle as an adaptive response to increasing energy demands after parturition (Ghaffari et al. 2019c). This was further confirmed by elevated levels of protein and mRNA abundance of branched-chain α-keto acid

dehydrogenase E1α (BCKDHA) in the scAT of HBCS versus NBCS cows prepartum, pointing to an increased oxidative capacity for irreversible degradation of transamination products of branched-chain amino acids (BCAA) in the scAT of overconditioned cows (Webb et al. 2020b). In addition, elevated concentrations of biogenic amines (BA) in serum and skeletal muscle in HBCS than NBCS cows led to the suggestion that overconditioning might result in altered BA metabolism in the periparturient period (Ghaffari et al. 2019b).

The combination of preselection based on body condition parameters and differential feeding prior to dry-off resulted in cows differing in their intensity of lipid mobilization after calving; likely leading to increased release of metabolic and endocrine compartments into the circulation when comparing cows with high and normal lipid mobilization.

The differences in lipolysis between groups after parturition in our study might also have an impact on the adipocyte metabolism (Bélanger et al. 2002). Adipose tissue regulates lipid homeostasis and is involved in glucose metabolism, insulin sensitivity, and inflammatory processes through the action of several factors secreted by adipocytes, which are active cells that participate in endocrine, paracrine, autocrine, and intracrine mechanisms (Kershaw and Flier 2004). Adipose tissue also secretes numerous adipokines that regulate steroidogenesis in the adrenals and gonads and also contributes to local steroid hormone levels through locally converted and released steroids (Kershaw and Flier 2004; Campos et al. 2008). Obesity-related dysfunction of AT causing various malignancies and increased risk of metabolic disorders, including impaired insulin sensitivity, has already been shown in humans (Bélanger et al. 2006; Blüher 2009). Consequently, variations in the extent of AT mobilization may also lead to shifts in steroid metabolism in dairy cows with different body condition.

### ***5.2*** *Steroid concentration in blood versus scAT of dairy cows*

Studies in humans provided evidence that circulating steroid hormones originate from various AT sites (e.g., abdomen, breast; Szymczak et al. 1998), in addition to the “classical” sites such as gonads and adrenal gland. It has also been shown that steroids can be taken up by AT and metabolized locally by steroidogenic enzymes (Bolt and Gobel 1972; Bélanger et al. 2002; Li et al. 2015). Circulating adipokines secreted and synthesized by AT may also contribute to steroid levels by regulating steroidogenesis in the gonads and adrenals (Kershaw and Flier 2004; Li et al. 2015). In turn, locally produced sex steroids are involved in AT distribution, accumulation, and metabolism (Ahima and Flier 2000; Tchernof and

Després 2013). Differences in the body mass and pronounced AT growth in humans may also affect peripheral steroid metabolism (Bélanger et al. 2002).

In dairy cows, holistic studies of steroid metabolism focusing on AT metabolism are scarce, especially with respect to the extent of differential lipid mobilization after calving. In human studies, Deslypere *et al.* (1985) demonstrated that steroid concentrations in AT were up to 400 times higher than in total plasma. In order to compare blood and scAT steroid content for the results of the current study, we were considering a mean TBF of 102 kg and 48 kg for HBCS and NBCS cows 7 weeks a.p., respectively (based on BFT equivalents [BFT, HBCS: 2.04 cm; NBCS: 0.95 cm]: 10 mm BFT equals about 50 kg body fat; Schröder and Staufenbiel 2006). Furthermore, we considered a total blood volume (TBV) of 44 and 42 L for HBCS and NBCS cows, respectively, based on the results of Turner and Herman ((1931); 61 mL/kg BW for mature non-lactating cows [BW, HBCS: 727 kg; NBCS: 693 kg]). Hence, for comparison of blood and scAT steroid contents (e.g., progesterone) we proceeded as follows:

*Progesterone content in TBF [ng]=*

progesterone concentration in scAT [ng/g] * 1,000 * TBF (kg)

*Progesterone content in TBV [ng] =*

progesterone concentration in serum [ng/mL] * 1,000 * TBV (L)

Assuming that one liter of blood is equivalent to approximately one kilogram, the total progesterone content in the AT of HBCS versus NBCS cows 7 weeks a.p. was about 46- and 20-fold higher, respectively, than in serum. At parturition (1-week p.p.) these differences magnified to 112- and 77-fold higher steroid content in AT compared with plasma in HBCS and NBCS cows, respectively, indicating local uptake and accumulation of steroids from the bloodstream in the bovine AT, and also demonstrating the influence of differences in body condition on steroid accumulation in the AT.

The precursor steroid DHEA-S was undetectable in the serum of the cows in our study, but was among the most highly accumulated steroids in scAT, highlighting the importance of local steroid conversion from steroid precursors (Bélanger et al. 2002). Adrenal precursor steroids such as DHEA, DEAH-S, and androstenedione have a major importance in the formation of biological active estrogens in peripheral tissues of women (Labrie 1991) and

presumably also in dairy cows, since these three precursor steroids had higher concentrations in the scAT versus serum in our study. In addition, the concentrations of sex steroids and their precursors in scAT (e.g., progestins, androgens, but not estrogens) were significantly and positively correlated with serum sex steroids, independent of grouping and timepoint. Nevertheless, when pregnancy-related changes in sex steroids are considered, progestins in scAT and serum were highly correlated in peak lactation but not during late pregnancy, indicating saturation of fat with progestins (especially progesterone), reflecting increased concentrations of these steroids over long periods (e.g., pregnancy; Hamudikuwanda et al. 1996).

### ***5.3*** *Differences in the steroid concentration in blood and scAT relative to periparturient body condition changes of cows*

The conversion of inactive C19 precursor steroids to active androgens or estrogens in peripheral target tissue depends on the expression and action of steroidogenic enzymes regulating the local amount of active steroids (Labrie 1991). The steroidogenic enzymes HSD3B1 and HSD17B12 are both involved in the formation of androgens (Labrie 1991). The hepatic mRNA abundance of HSD3B1 was increased in HBCS versus NBCS cows one week after parturition, along with higher circulating progesterone, pregnenolone and androsterone, indicating amplified hepatic formation of these steroids in overconditioned cows. Conversely, the increased concentration of progestins in the blood of HBCS compared with NBCS cows may be related to the release of stored steroids from AT mobilization in early lactation, as demonstrated previously in non-lactating cows (Rodrigues et al. 2011).

In mammals, pregnenolone – which is synthesized mainly in the gonads and adrenal glands – is known to be the principal precursor of all steroid hormones and is the main steroid synthesized from cholesterol, initiated by StAR and CYP11A1 (Li et al. 2015; Li et al. 2014). On the other hand, pregnenolone taken up from the circulation can be converted to 17-OHP by CYP17A1 (Li et al. 2015). In the present study, StAR mRNA abundance was not affected by the group, and neither CYP11A1 nor CYP17A1 were measured in this study; therefore, the origin of higher circulating pregnenolone concentrations is unknown, but could originate from increased mobilization and release from the AT in HBCS compared with NBCS cows in early lactation. In addition, peripartum NEFA concentrations were negatively correlated ($-0.4 < r < -0.7$; $P < 0.05$) with progestins and androgens in blood and adipose tissue, regardless of group and time. However, these associations were more pronounced when only HBCS

cows were considered, indicating the release of steroids from AT as a result of the enhanced lipomobilization in HBCS cows.

The conversion of active 17-ß-hydroxy and inactive 17-keto steroids is catalyzed by HSD17 enzymes and plays an essential role in the final steps of androgen and estrogen biosynthesis. Different HSD17 types catalyze either reductive (e.g., E1 to E2; types 1, 5, 7 and 12) or oxidative pathways (e.g., E2 to E1; types 2, 4, 10, and 11; Bellemare et al. 2009; Hetemäki et al. 2017; Tchernof et al. 2015). In our study, we investigated the mRNA abundance of type 12 HSD17, which is supposed to catalyze the synthesis of E2 from E1 (Luu-The et al. 2006; Blanchard and Luu-The 2007). Furthermore, HSD17B12 has been reported to be the most abundant steroid-converting enzyme in human AT (Li et al. 2015; Blouin et al. 2009). It is also involved in the elongation process of VLCFA, highly expressed in organs related to lipid metabolism (e.g., liver, kidney, heart and skeletal muscle; Moon and Horton 2003; Sakurai et al. 2006). It has been proposed that HSD17B12 is a species- and concentration-dependent multi-substrate enzyme that catalyzes the elongation of fatty acids as well as the transformation of sex steroids, depending on substrate availability (Bellemare et al. 2009). The HSD17B12 mRNA abundance was increased in the scAT of HBCS versus NBCS cows 7 weeks a.p. ($P < 0.05$), whereas the scAT concentrations of androgens and estrogens were numerically increased only in HBCS cows at that timepoint. Nevertheless, HSD17B12 mRNA abundance in scAT was moderately correlated with androgens and progestins in scAT, suggesting that HSD17B12 converts androgens rather than estrogens.

In AT, gene expression of estrogenic HSD17 enzymes was reported to be lower than those of the androgenic ones; therefore, androgen biosynthesis may be more relevant than estrogen biosynthesis in AT (Blouin et al. 2009). Assuming that the HSD17B12 enzyme converts androgens rather than estrogens, the higher E1 concentration in scAT compared with serum in cows of our study could also be due to local estrogen synthesis via the steroid sulfatase enzyme (STS; (Tchernof et al. 2015)) or CYP19 aromatization of androstenedione to E1 (Hetemäki et al. 2017), or both.

The STS expression or activity was not examined in the present work, whereas CYP19 mRNA was not detectable with the protocol used herein. Hydrolysis of estrone-sulfate (E1S) to E1 by STS takes place in various female AT depots (Hetemäki et al. 2017). Circulating E1S serves as a hormone reservoir, while sulfonated steroids often exceed the concentrations of free (unconjugated) steroids in the circulation and various tissues (Hetemäki et al. 2017; Schwarzenberger et al. 1993), which was also observed for DHEA-S in the scAT of cows in

our study. Sulfonated steroids are considered as important source for the local steroid formation (Labrie 1991). In addition, steroidogenic enzymes such as CYP17A1 and CYP11A1 were also shown to be able to convert sulfonated steroids in a comparable manner to the unconjugated ones, suggesting an alternative steroidogenic pathway for sulfonated steroids (Neunzig et al. 2014).

Another possible explanation for the increased expression of HSD17B12 mRNA in HBCS cows in our study might be that the enzyme is related to lipid metabolism (Sakurai et al. 2006) rather than steroid conversion. Correspondingly, higher hepatic abundance of HSD17B12 mRNA in NBCS than in HBCS after parturition may point to the same mechanism, as positive energy balance was achieved earlier in NBCS cows, likely leading to lipogenic processes. Negative correlations of circulating NEFA with scAT HSD17B12 mRNA expression ($r = -0.6$; $P < 0.001$; independent of group and time) may support this assumption. Nevertheless, local differences in HSD17 enzyme expression have also been reported (Payne and Hales 2004), suggesting that other AT depots may also have differential HSD17 enzyme expression compared with scAT.

The GC are also secreted by the AT and act through paracrine or autocrine mechanisms (Ahima and Flier 2000) by regulating carbohydrate and amino acid metabolism, modulating stress and inflammatory responses, maintaining blood pressure, and being part of the feedback mechanism in the immune system (Munck and Náray-Fejes-Tóth 1992). The GC are mainly produced in the adrenal glands and regulate the supply with energy from endogenous energy stores (Tataranni et al. 1996). In metabolic disorders such as insulin resistance or obesity-related diabetes, the main biological role of the GC/glucocorticoid receptor axis is to suppress inflammatory processes and control energy homeostasis (de Guia et al. 2014; Rose and Herzig 2013).

In our study, enhanced lipomobilization in HBCS compared with NBCS after parturition was associated with decreased concentrations of the following GC in scAT: 11-DOC, corticosterone, cortisone, and cortisol. However, this was not followed by increased GC in circulation. Local formation of GC from the precursor steroids progesterone and 17-OHP is triggered by the enzyme CYP21. Considering that CP21 was present in bovine scAT in our study and increased mRNA abundance of the enzyme was detected in peak lactation regardless of grouping, indicates that local formation of GC was induced. Under normal fasting conditions, lipolysis in the AT is promoted by GC that increase the activity of important lipases and inhibit the activity of lipoprotein lipase (Peckett et al. 2011; Yu et al.

2010). The higher GC concentration in the scAT of NBCS compared with HBCS cows might be related to functions associated with lipogenesis, because NBCS cows went into positive energy balance earlier than HBCS cows, suggesting that GC are involved in lipogenic processes exponentiating the lipogenic function of insulin by regulating the expression of important genes within this pathway (Hillgartner et al. 1995).

Interestingly, circulating cortisol also tended to be higher in NBCS than HBCS across all measured time points, but was within the range reported for dairy cows during the periparturient period (Fustini et al. 2017), indicating increased gluconeogenesis, as NBCS cows also had greater feed intake during the periparturient period. Cortisol is mainly produced by the *Zona fasciculata* of the adrenal cortex and due to its catabolic action provides the body with energy-rich compounds from endogenous stores (Tataranni et al. 1996). In addition, cortisol is crucial for adipocyte differentiation, and the activation of cortisone to cortisol is regulated by the enzyme HSD11B1 (Tomlinson et al. 2004). In dairy cows, the enzyme activity of HSD11B1 was measured in different AT depots, being located in mature and already differentiated adipocytes, also indicating an anti-inflammatory effect of cortisol in bovine AT (Friedauer et al. 2015). In the present study, the mRNA abundance of HSD11B1 in scAT peaked at week 1 p.p., regardless of grouping, indicating a role of parturition in the enzyme expression in scAT. Because parturition is associated with inflammatory processes (Bradford et al. 2015), different GC concentrations in scAT between groups in our study could be related to acute inflammatory responses, since GC have immunosuppressive and anti-inflammatory effects on multiple organs, including AT (Munck and Náray-Fejes-Tóth 1992; Lee et al. 2011; Coutinho and Chapman 2011).

On the other hand, comprehensive investigations on childhood obesity demonstrated that mineralocorticoids and their metabolites were down regulated in the urine of obese children compared to control children (Vitkin et al. 2014). The authors suspected that this phenomenon, which is related to obesity and the metabolic syndrome in humans, is a defect in cortisol degradation through inhibition of cortisol-metabolizing enzymes and an alteration in the renin-angiotensin-aldosterone system leading to increased mineralocorticoid receptor activity (Vitkin et al. 2014). In addition, mineralocorticoid deficiency may be associated with enhanced conversion of androgens by CYP17A1, contributing to hyperandrogenism in obese women (Hirsch et al. 2012; Vitkin et al. 2014). The latter observations are consistent with the results of the current study, in which overconditioned cows had lower GC and higher androgen concentrations in scAT and blood

compared with normal conditioned cows. Unfortunately, CYP17 was not detectable with the protocol used herein, suggesting that its mRNA abundance was generally too low.

The final product of *de novo* adipose steroidogenesis has been proposed to be 11-deoxycorticosterone in humans (MacKenzie et al. 2008), but corticosterone in rodents (van Schothorst et al. 2005), because of the absence of CYP17A1 mRNA in AT in both studies. Still, CYP17A1 mRNA expression and enzymatic activity has been detected in female abdominal AT by one research group (Puche et al. 2002), but others failed to detect this enzyme in human scAT and abdominal AT (Dalla Valle et al. 2006; MacKenzie et al. 2008; Wang et al. 2012), which might be explained by the low expression of CYP17A1 in AT and high variation between different AT samples (Li et al. 2015).

The liver is the main organ for the activation of GC. In the present study, the hepatic mRNA abundance of HSD11B1 was up to 169-fold higher compared with scAT, irrespective of group, indicating an important role in hepatic activation of GC in dairy cows. Moreover, hepatic mRNA of HSD11B1 was increased in HBCS compared with NBCS cows 7 weeks before calving. It has been suggested that local steroid metabolism only alters the amounts for specific needs without affecting circulating concentrations (Masuzaki et al. 2001), and that it is not clear whether increased HSD11B1 expression in AT is a consequence or the cause of obesity in humans (Li et al. 2015). Though, local overexpression of HSD11B1 resulted in higher cortisol concentrations in murine AT, which were associated with visceral obesity, development of metabolic syndrome, hypertension, and diabetes (Masuzaki et al. 2001). Furthermore, overexpression of HSD11B1 in liver and AT has been demonstrated in genetically obese rats (Walters et al. 2002) and obese humans (Bulun and Simpson 1994), suggesting a role for this enzyme in lipid metabolism and adipogenesis (Tomlinson et al. 2004).

Leptin and other cytokines have been described to upregulate HSD11B1 activity in human adipocytes (Tomlinson et al. 2004). Although increased hepatic mRNA abundance of HSD11B1 in overconditioned cows in our study was accompanied by higher leptin serum concentration during late gestation (leptin in HBCS cows up to 2.8-fold higher compared to NBCS cows), hepatic HSD11B1 abundance and leptin were not correlated.

The presence of StAR and CYP11A1 enables a cell in producing steroids *de-novo* (Li et al. 2014). In our study, the mRNA abundance of StAR peaked in bovine scAT 12 weeks *post partum*, irrespective of group. In murine adipocytes, mRNA abundance of StAR was several-

fold higher during adipogenesis (Li et al. 2014), indicating increased cholesterol uptake into the inner mitochondrial membrane in peak lactation when cows enter a positive nutrient balance and a restart of adipogenesis occurs. However, CYP11A1 mRNA was undetectable in bovine scAT with the protocol used in the current work, and studies in murine cell cultures imply that CYP11A1 appears to play only a minor metabolic role in adipocyte steroidogenesis (Li et al. 2014). Therefore, the detection of StAR in scAT in our study may reflect other processes such as *de novo* synthesis of oxysterols, which also play a relevant role in adipose steroidogenesis (Li et al. 2014). When comparing different AT depots or sites, metabolic processes (e.g., metabolism of fatty acids, proteins, or hormones) are altered with respect to lipolysis, which is due to homeorhetic regulations in the AT (Jensen 2008; Locher et al. 2012; Singh et al. 2014; de Koster et al. 2016). Thus, steroid hormone metabolism in AT, which is mediated by the presence of locally acting steroidogenic enzymes, also depends on local differences (Payne and Hales 2004). For instance, StAR mRNA expression has been shown to be 10-fold higher in human omental AT than in scAT (MacKenzie et al. 2008). Consequently, different AT depots (omental, mesentery, mammary) in dairy cows are also expected to have different conditions with respect to steroidogenesis.

Interestingly, Chen *et al.* (2012) revealed that gene expression of steroidogenic enzymes can also be altered by changes in the local amount of approaching steroids; for example, increasing concentrations of androstenone, acting as pheromone (in pigs it is a component of boar taint), stimulates HSD17B7 gene expression in porcine primary hepatocytes. Furthermore, the catalytic function of HSD17B7 in hepatocytes appears to differ from its actions in other organs (Chen et al. 2012). This is another example of how the complexity of steroid metabolism is species-, sex-, and tissue-specific (Payne and Hales 2004; Dalla Valle et al. 2006). The expression of steroidogenic enzymes is related to a variety of transcription factors, whereas the interaction between steroids and steroidogenic enzymes is poorly understood so far (Payne and Hales 2004; Chen et al. 2012).

With regard to a holistic evaluation of the steroid hormone metabolism in dairy cows, differing in their lipid mobilization after calving, our aim was also to analyze steroid levels in milk, which unfortunately has not been possible so far due to storage problems and acidification of the collected milk samples. However, the feasibility of measuring the steroid content in the milk samples has not yet been finalized and may be published in later works.

For decades, milk hormone levels (e.g., progesterone) have been used to reflect *post partum* luteal function and ovulation (ovarian cyclicity) in cattle (Pope and Swinburne 1980). The very high correlation of milk and systemic blood levels of progesterone and estradiol-17ß during the estrous cycle in *post partum* dairy cows(r = 0.87 and 0.85, respectively; Abeyawardene et al. 1984) makes hormone-related studies more convenient and less invasive for animals when milk hormone levels are determined. In addition, whole milk concentrations of steroids such as progesterone, estradiol-17ß (Erb et al. 1977), and androstenedione (Gaiani et al. 1984) have been found to exceed those in circulation severalfold during different stages in gestation, making this body fluid an important determinant for a holistic assessment of steroid metabolism.

### *5.4 The effect of parity on the steroid metabolism in dairy cows*

In dairy cows, periparturient endocrine and metabolic changes are influenced by extensive lipid mobilization interacting with the parity number (Meikle et al. 2004; Gärtner et al. 2019). In the present study, parity of the cows affected 6 of 19 and 9 of 17 of the steroids in serum and scAT, respectively, regardless of group and time point. The mRNA abundance of the steroidogenic enzymes HSD17B12 and HSD3B1 was also affected by parity; here, cows with more than 3 parities showed higher enzyme mRNA abundance compared to cows in second parity. Conversely, second parity cows had higher concentrations of steroid hormones in blood (e.g., DHEA, DHT, and estrone) and scAT (e.g., androstenedione, DHEA, and estradiol) compared with multiparous cows (≥ 4 parities), which was also observed in terms of progesterone concentration and secretion by others (Carvalho et al. 2014; Hori et al. 2019). The decreased steroid hormone concentration in multiparous cows in our study may exert an age-related effect on luteal function (Hori et al. 2019), as indicated by an increased rate of pregnancies per AI as shown in primiparous versus multiparous cows (Carvalho et al. 2014). It is well known that elevated body condition loss (Cardoso et al. 2013; Carvalho et al. 2014) or excessive lipolysis and ketogenesis (Leroy et al. 2005) in the first weeks after calving can lead to impaired fertility in dairy cows.

As already discussed above (chapter 5.1), the fertility traits calculated on a retrospective basis in the cows of our study showed no differences between parities and were only numerically increased in HBCS (23-days longer CI and 0.4-times more AI) compared to NBCS cows, presumably as a result of the limited number of animals in this study.

To date, only certain parameters of the complex network of the steroid metabolism in dairy cows have been selectively highlighted (e.g., sex steroids) or the analysis has been limited to specific segments (e.g., blood, milk, or AT). To our knowledge, local steroid metabolism in AT (and measurement of steroidogenic enzyme abundance) in cows with different degrees of *post partum* tissue mobilization and comparison of steroid concentration in serum and AT have not been studied previously.

Therefore, the results of the present work provided the first comprehensive overview of periparturient (-7, 1, 3, and 12 weeks relative to parturition) concentrations of steroid hormones in blood (19 steroids) and AT (17 steroids), together with the measurement of the abundance of 5 steroidogenic enzymes in liver and AT of cows differing in their degree of *post partum* lipolysis. The results indicate that over-conditioned cows had an aberrant steroid pattern compared to normal conditioned cows, which is likely due to the difference in *post partum* metabolism and lipolysis between the two groups of cows, but requires further investigation.

# 6 Conclusions and future perspectives

The experimental approach used in this work provided a model for dairy cows that allows for comparing animals with different intensities of lipid mobilization after parturition. Cows that were over-conditioned before calving were mobilizing more body reserves after calving and were metabolically more challenged compared to normal conditioned cows. In addition, high BCS at calving was associated with impaired antioxidative capacity and greater insulin and glucose concentrations indicating IS. The steroid profile in scAT as well as the expression of steroidogenic enzymes was altered between the two groups likely as a result of different lipomobilization rates during the periparturient period, which was also influenced by the parity of the cows. The higher GC concentration in scAT of NBCS versus HBCS cows may indicate that GC are involved in lipogenic processes during peak lactation. However, the differences in the GC content in scAT between groups might also be related to inflammatory processes, which needs to be validated by further studies.

The results of this perspective study provide evidence that the interconversion of mineralo- and glucocorticoids as well as androgens, initiated by the steroidogenic enzymes CYP21 and HSD17B12, occurs in bovine scAT. However, the formation of specific steroids could not be attributed with certainty to the respective enzymes, which might be investigated by further studies involving the measurement of steroid receptors or protein activity of the respective steroidogenic enzymes. Moreover, a holistic approach of the steroid metabolism in dairy cows including other matrices (e.g., milk, urine, feces) and adipose depots (e.g., visceral, omental, mammary) along with the determination of steroid metabolomics would increase the overall understanding of the steroid metabolism in the dairy cow. The mechanism of the regulation of steroidogenic enzymes and expression patterns with respect to changes in tissue mobilization in dairy cows also requires further investigation.

## 7 Summary

The adipose tissue (AT) in dairy cows undergoes tremendous changes during the periparturient period. The energy stored as triglycerides during late gestation and the dry period is needed for the rapidly increasing energy requirements in early lactation, when energy intake cannot meet the energy needs of the animals. As a result, these reserves are mobilized to the extent that the energy balance (EB) turns to negative values. The release of fatty acids from AT mobilization during times of energy deficiency can be used by various organs (e.g., brain, heart, liver, and mammary gland) to generate energy or to form other functional compounds (e.g., milk fat). When dairy cows are over-conditioned before calving, the mobilization of body reserves *post partum* is more pronounced, with a more severe and persistent negative EB compared to normal conditioned cows. Therefore, over-conditioned cows are more metabolically challenged than normal conditioned cows and have a higher propensity to develop metabolic disorders and oxidative stress.

The AT is able to take up and store lipophilic steroids from the circulation and metabolize steroids locally by steroidogenic enzymes. In humans, it has been described that increasing blood concentrations of steroids may be related to obesity and local fat accumulation. In addition, local production of sex steroids in AT has been implicated as a significant factor of body fat metabolism, whereby locally produced steroids contribute to total steroid levels and may regulate AT metabolism at a functional level. Consequently, steroids may be released into the circulation along with metabolites during periods of body fat mobilization. Thus, enhanced lipolysis in over-conditioned cows after calving may result in increased release of steroids from AT and alter the steroid profile compared to normal conditioned cows. Thus, the aims of the present work were to (1) investigate the extent of pronounced (HBCS) or moderate (NBCS) mobilization of body reserves on changes in production and selected metabolic and endocrine parameters in dairy cows from late pregnancy to subsequent lactation, and (2) to compare changes in steroid hormone concentrations in serum and subcutaneous AT (scAT) during this time in HBCS and NBCS cows, and (3) to quantify the mRNA expression of various steroidogenic enzymes in liver and scAT and compared these expressions between HBCS and NBCS cows.

In the first part of the study (Manuscript I), we established the animal model required for our objectives by obtaining cows with either high or normal mobilization of body reserves after calving for the targeted comparison. We then characterized performance, metabolic,

and endocrine changes from 7 weeks *ante partum* (a.p.) to 12 weeks *post partum* (p.p.) of the HBCS and NBCS cows. The pregnant and multiparous Holstein cows (n = 38) were assigned to two groups based on their actual body condition score (BCS) and back fat thickness (BFT) 15 weeks before calving and fed different energy levels from 15 to 7 weeks a.p. (HBCS: 7.2 NEL MJ/kg dry matter (DM); NBCS: 6.8 NEL MJ/kg DM) to achieve the target BCS and BFT at dry-off (HBCS: >3.75 and >1.4 cm; NBCS: <3.5 and <1.2 cm, respectively). The allocation of cows was also based on differences in BCS and BFT (predisposition) in the previous and the ongoing lactation with comparable milk yields between the two groups (HBCS: 10,315 ± 437 kg; NBCS: 10,361 kg ± 302 kg; as means ± SEM). During the dry-off period and the following lactation period, both groups received identical diets. In addition to monitoring BCS, BFT, and body weight of cows, blood samples were harvested weekly from 7 weeks a.p. to 12 weeks p.p. to determine the serum concentrations of metabolites and metabolic hormones.

At dry off, the groups reached the targeted difference in BCS and BFT (as means ± SEM): HBCS with 3.8 ± 0.09 and 2.0 ± 0.11, respectively, and NBCS with 3.0 ± 0.05 and 0.9 ± 0.07, respectively. Moreover, body condition differed between groups throughout the observation period ($P < 0.001$); here, HBCS cows had consistently higher BCS, BFT, and BW than NBCS and lost twice as much BFT during the first 7 weeks p.p. compared with NBCS cows. The greater reduction in body mass in HBCS was accompanied by increased concentrations of NEFA and BHB in serum after calving than in NBCS, indicating increased lipomobilization and ketogenesis due to the lower feed intake in HBCS relative to NBCS cows. Consequently, EB was more negative in HBCS than in NBCS cows, and NBCS reached positive energy balance about two weeks earlier than HBCS cows. Elevated mean concentrations of circulating insulin in HBCS relative to NBCS cows at all measured time points indicated reduced insulin sensitivity. In addition, overconditioning before calving was associated with impaired antioxidative capacity (increased oxidative stress), as reflected by lower plasma ferric iron reduction capacity (FRAP).

The results of the first manuscript showed that the experimental design was appropriate to study different intensities of lipid mobilization in dairy cows during the *post partum* period: the combination of pre-selection according to body condition parameters together with differential feeding before dry-off promoted the difference in the intensity of mobilization of body reserves accompanied by increased changes in blood metabolites and hormones when comparing both groups.

As an active endocrine organ, the AT is an important site of steroid hormone metabolism where steroids are stored and locally converted to other steroids by specific steroidogenic enzymes. For the second manuscript, we hypothesized that augmented lipid mobilization after calving in over-conditioned cows results in greater release of steroids from AT and contributes to alterations in the steroid profile in blood and AT compared to normal conditioned cows. To gain insights into possible interactions between the expression of regulatory enzymes involved in steroid biosynthesis and steroid levels during the periparturient period, the steroid concentrations in blood and AT of the two groups were assessed by a targeted metabolomics approach (LC-MS/MS) quantifying 19 different steroids. In parallel, the mRNA abundance of five steroidogenic enzymes involved in the main pathways of steroid metabolism (steroidogenic acute regulatory protein (StAR), 17 ß-hydroxysteroid dehydrogenases type 12 (HSD17B12), 11 ß-hydroxysteroid dehydrogenase type 1 (HSD11B1), 3 β-hydroxysteroid dehydrogenase type 1 (HSD3B1), and steroid 21-hydroxylase (CYP21)) was determined in liver and scAT by qPCR. For this, liver and subcutaneous AT (scAT; tailhead) were biopsied at -7, 1, 3, and 12 weeks relative to parturition. In addition, from 7 weeks a.p. until 12 weeks p.p., serum samples were analyzed for weekly progesterone concentration using an enzyme-linked immunosorbent assay.

Our results showed that steroid concentrations in scAT were several-fold higher than in circulation (based on calculations of body fat content and blood volume). Irrespective of time and grouping, progestins and androgens in the circulation were strongly correlated ($r > 0.7$; $P < 0.001$) with the respective steroids stored in scAT. During the *post partum* period, concentrations of cortisol, corticosterone, and cortisone were up to 3.5-fold higher in the scAT of NBCS relative to HBCS cows ($P < 0.05$). One week after parturition, circulating progesterone, androsterone, and pregnenolone were higher in HBCS than in NBCS cows ($P < 0.05$).

Our data provided evidence that local conversion of androgens and mineralo- and glucocorticoids is initiated by CYP21 and HSD17B12 in bovine scAT. Across time points, cow parity influenced 6 of 19 and 9 of 17 of the steroid concentrations in serum and scAT, respectively. Cows with ≤ 3 parities had higher steroid concentrations compared to cows with more than 4 parities. However, compared with second-parity cows, multiparous cows (≥ 4 parities) showed higher mRNA abundance of the steroidogenic enzymes HSD3B1 (scAT and liver) and HSD17B12 (scAT), suggesting that both enzyme expression and steroid levels are differentially affected by parity number.

The higher glucocorticoid concentration in the scAT of NBCS cows compared with HBCS cows after parturition may either influence adipogenesis by differentiation of preadipocytes into mature adipocytes or act as an acute inflammatory response mechanism, or both. The results from our observations contribute to a better understanding of the complex regulation of periparturient steroid metabolism and local steroid conversion in the AT of dairy cows.

Cows that were over-conditioned at calving were more metabolically challenged during the early lactation period, with intense mobilization of body fat and increased blood concentrations of metabolites and hormones compared with normal conditioned cows. In addition, over-conditioned cows showed altered steroid profiles in AT and changes in steroidogenic enzyme mRNA abundance patterns compared to NBCS cows, which was further influenced by cow parity. Further publications derived from the experimental setup established here deepened the understanding of the pathophysiological sequelae of over-conditioning on a molecular basis (metabolic footprint). However, to depict the holistic steroid metabolism in the dairy cow, further research is needed involving additional factors contributing to local steroid formation (e.g., further steroidogenic enzymes, steroid receptors, steroid precursors, sulfonated steroids) as well as further body fluids such as urine and milk, and also feces or additional target tissues (e.g., different AT sites, ovary).

# 8 Zusammenfassung

Das Fettgewebe (AT) von Milchkühen unterliegt im geburtsnahen Zeitraum enormen Veränderungen. Die während der späten Trächtigkeit und Trockenstehzeit als Triglyceride gespeicherte Energie wird für den schnell steigenden Energiebedarf in der Frühlaktation benötigt. Während dieser Periode kann der Energiebedarf der Tiere oftmals nicht durch die Energie-(Futter)-aufnahme gedeckt werden. Infolgedessen werden die Energiereserven in einem Ausmaß mobilisiert, welches die Energiebilanz (EB) in den negativen Bereich absinken lässt. Fettsäuren, die bei Energiemangel aus der Fettmobilisierung freigesetzt werden, können von verschiedenen Organen (z.B. Gehirn, Herz, Leber und Milchdrüse) zur Energiegewinnung oder zur Bildung anderer funktioneller Verbindungen (z.B. Milchfett) genutzt werden. Sind Milchkühe zur Kalbung hin überkonditioniert, findet eine ausgeprägtere Mobilisierung von Körperfettreserven nach der Kalbung statt, verbunden mit einer stärkeren und länger anhaltenden negativen EB im Vergleich zu normal konditionierten Kühen. Die vermehrte Freisetzung von Fettsäuren, die aus einer erhöhten Körperfettmobilisierung bei überkonditionierten Kühen im Vergleich zu normal konditionierten Kühen resultiert, verursacht eine stärkere metabolische Belastung und führt vermehrt zu Stoffwechselstörungen und oxidativem Stress.

Das AT kann lipophile Steroide aus dem Blutkreislauf aufnehmen, abspeichern und lokal durch steroidogene Enzyme metabolisieren. Eine erhöhte Steroidkonzentrationen im Blut wurden zudem mit der Entwicklung von Adipositas und lokaler Fettansammlung beim Menschen in Verbindung gebracht. Darüber hinaus wurde die lokale Produktion von Sexualsteroiden im Fettgewebe als ein wichtiger Bestandteil des humanen Körperfettstoffwechsels beschrieben, wobei die lokal produzierten Steroide zum Gesamtsteroidspiegel beitragen und den AT-Stoffwechsel auf funktioneller Ebene regulieren. Folglich können Steroide während einer Körperfettmobilisierung zusammen mit Metaboliten in den Blutkreislauf abgegeben werden. Eine erhöhte postpartale Lipolyse bei überkonditionierten Kühen kann demnach zu einer erhöhten Freisetzung von Steroiden aus dem AT führen und das Steroidprofil im Vergleich zu normal konditionierten Kühen verändern.

Die Ziele der vorliegenden Arbeit waren daher, (1) das Ausmaß einer ausgeprägten (HBCS) bzw. moderaten Mobilisierung (NBCS) von Körperreserven auf Veränderungen der Milchleistung und ausgewählter metabolischer und endokriner Parameter bei Milchkühen

von der späten Trächtigkeit bis zur nachfolgenden Laktation zu untersuchen, und (2) Veränderungen in der Steroidhormonkonzentration im Blut und subkutanem AT (scAT) während dieser Zeit zwischen HBCS- und NBCS-Kühen zu vergleichen sowie (3) die mRNA-Expression verschiedener steroidogener Enzyme in Leber und AT-Gewebe zu quantifizieren und diese zwischen HBCS- und NBCS-Kühen zu vergleichen.

Im ersten Teil der Studie (Manuskript I) wurde das für unsere Zielsetzung erforderliche Tiermodell etabliert. Für den beabsichtigten Vergleich wurden Kühe mit ausgeprägter bzw. moderater Mobilisierung von Körperreserven nach dem Kalben gewählt. Anschließend wurden in den beiden Gruppen die Unterschiede in der Milchleistung sowie in metabolischen und endokrinen Parametern im Zeitraum von 7 Wochen vor (a.p.) bis 12 Wochen nach der Kalbung (p.p.) charakterisiert. Trächtige und multipare schwarzbunte Holsteinkühe (n =38) wurden 15 Wochen vor dem erwarteten Abkalben anhand ihrer Körperkondition (BCS) und Rückenfettdicke (BFT) in zwei Gruppen eingeteilt und erhielten von 15 bis 7 Wochen a.p. eine unterschiedlich intensive Energiezufuhr (HBCS: 7,2 NEL MJ/kg Trockensubstanz (DM); NBCS: 6,8 NEL MJ/kg DM) um die geplanten Körperkonditionsziele (HBCS: BCS >3,75 und BFT>1,4 cm; NBCS: BCS <3,5 und BFT <1,2 cm) zum Trockenstellen (7 Wochen a.p.) zu erreichen. Die Einteilung der Kühe basierte außerdem auf Unterschieden des Körperkonditionsverlaufs (BCS/BFT; Prädisposition) der vorherigen und gegenwärtigen Laktation, wobei die Milchleistung zwischen beiden Gruppen vergleichbar war (NBCS: 10.361 kg ± 302 kg; HBCS: 10.315 ± 437 kg; Mittelwert ± SEM). Während der Trockenstehzeit und der anschließenden Laktation erhielten beide Gruppen identische Futterrationen. Neben der Erfassung von BCS, BFT und Körpergewicht wurden wöchentliche Blutproben von 7 Wochen a.p. bis 12 Wochen p.p. entnommen, um die Serumkonzentration von ausgewählten Metaboliten und Stoffwechselhormonen zu bestimmen. Zum Zeitpunkt des Trockenstellens erreichten beide Gruppen die angestrebten Körperkonditionsziele (nachfolgend Mittelwerte ± SEM): HBCS mit 3,8 ± 0,09 BCS und 2,0 ± 0,11 BFT; NBCS mit 3,0 ± 0,05 BCS und 0,9 ± 0,07 BFT. Darüber hinaus unterschied sich Körperkondition zwischen den Gruppen während des gesamten Bobachtungszeitraums ($P < 0.001$); hier hatten HBCS-Kühe einen durchweg höheren BCS und BFT im Vergleich zur NBCS Gruppe und verloren während der ersten 7 Wochen p.p. im Schnitt doppelt so viel BFT verglichen mit den NBCS Kühen. Der erhöhte Körpermasseverlust in der HBCS- gegenüber der NBCS-Gruppe nach der Kalbung, war mit erhöhten Konzentrationen von NEFA und BHB im Serum verbunden, welches auf eine erhöhte Lipomobilisierung und Ketogenese aufgrund der geringeren

Futteraufnahme bei HBCS im Vergleich zu NBCS-Kühen hinwies. Infolgedessen zeigten HBCS-Kühe eine ausgeprägtere negative EB im Vergleich zu NBCS-Kühen, wobei NBCS-Kühe etwa zwei Woche früher eine positive Energiebilanz erreichten als HBCS-Kühe.

Zu allen gemessenen Zeitpunkten war die zirkulierende Insulinkonzentration in den HBCS-Tieren erhöht, was auf eine reduzierte Insulinsensitivität im Vergleich zu den NBCS-Tieren hinwies. Eine Überkonditionierung vor dem Kalben war mit einer verringerten oxidativen Kapazität (erhöhtem oxidativem Stress) verbunden, was sich in einer geringeren Plasma-Eisen-Reduktionsfähigkeit (FRAP) widerspiegelte. Die Ergebnisse des ersten Manuskripts zeigten, dass das Versuchsdesign geeignet war, unterschiedliche Intensitäten der Körperfettmobilisierung bei Milchkühen während der postpartalen Periode zu untersuchen: die Kombination aus Vorselektion des Körperkonditionsverlaufs und einer differenzierten Fütterungsintensität bis zum Trockenstellen führte vermutlich zu einem verstärkten Unterschied der Mobilisierung von Körperreserven zwischen den Gruppen, was durch erhöhte Unterschiede von Blutmetaboliten und Hormonen bestätigt wurde.

Als aktives endokrines Organ ist das AT ein wichtiger Ort des Steroidhormonstoffwechsels, in dem Steroide gespeichert und lokal durch spezifische steroidogene Enzyme in andere Steroide umgewandelt werden. Für den zweiten Teil der Studie (Manuskript II) stellten wir die Hypothese auf, dass bei überkonditionierten Kühen eine verstärkte Lipidmobilisierung nach dem Kalben zu einer höheren Freisetzung von Steroiden aus dem AT führt und somit zu einem veränderten Steroidprofil im Blut und AT im Vergleich zu normal konditionierten Kühen beiträgt. Um Einblicke in mögliche Zusammenhänge zwischen der Expression von regulatorischen, an der Steroidbiosynthese beteiligten Enzymen und dem Steroidspiegel während der peripartalen Periode von Milchkühen zu erhalten, wurde die Steroidkonzentrationen in Blut und AT der beiden Gruppen durch einen spezifischen Metabolomics-Ansatz (LC-MS/MS) quantifiziert, in dem 19 Steroide im Blut und 17 Steroide im AT analysiert wurden. Parallel dazu wurde die mRNA-Abundanz von fünf steroidogenen Enzymen, die an den wesentlichen Synthesewegen des Steroidstoffwechsels beteiligt sind (Steroidogenes akutes regulatorisches Protein (StAR), 17 ß-Hydroxysteroid-Dehydrogenase Typ 12 (HSD17B12), 11 ß-Hydroxysteroid-Dehydrogenase Typ 1 (HSD11B1), 3 β-Hydroxysteroid Dehydrogenase Typ 1 (HSD3B1) und Steroid 21-Hydroxylase (CYP21)), mittels qPCR in der Leber und im AT bestimmt. Hierfür wurden in den Wochen -7, 1, 3 und 12 relativ zur Kalbung Gewebebiopsien von der Leber und vom subkutanem AT (scAT; Schwanzansatz) entnommen. Zusätzlich wurde die Progesteron Konzentration im Serum auf

einer wöchentlichen Basis (7 Wochen a.p. bis 12 Wochen p.p.) mittels enzymgekoppelten Immunadsorptionstest (ELISA) analysiert.

Unsere Ergebnisse zeigten, dass die akkumulierte Steroidkonzentrationen im scAT um ein Vielfaches höher waren als in der Zirkulation (basierend auf Berechnungen des Körperfettgehalts und des Blutvolumens). Über den gesamten Beobachtungszeitraum hinweg wurde unabhängig von der Gruppierung ein starker Zusammenhang ($r > 0,7$; $P < 0.001$) zwischen den akkumulierten (scAT) und zirkulierenden Progestagenen und Androgenen festgestellt. Während der postpartalen Periode waren die Konzentrationen von Kortisol, Kortikosteron und Kortison im scAT der NBCS Gruppe bis zu 3.5-fach höher im Vergleich zur HBCS Gruppe ($P < 0.05$). Eine Woche nach der Kalbung waren die zirkulierenden Progesteron-, Androsteron- und Pregnenolon-konzentrationen bei HBCS Kühen höher als bei NBCS Kühen ($P < 0.05$). Die Ergebnisse dieser Arbeit zeigten außerdem, dass die lokale Umwandlung von Androgenen sowie von Mineralo- und Glucocorticoiden durch die Enzyme HSD17B12 und CYP21 im bovinen scAT initiiert wird. Außerdem beeinflusste die Parität der Kühe die Konzentration von 6 der 19 und 9 der 17 gemessenen Steroidode im Serum und scAT im peripartalen Zeitraum. Kühe mit Parität (Kalbungen) ≤ 3 hatten höhere Steroidkonzentrationen im Vergleich zu Kühen mit mehr als 4 Kalbungen. Im Vergleich zu Kühen der zweiten Parität zeigten Kühe mit mehr als 4 Kalbungen jedoch eine höhere mRNA-Abundanz der steroidogenen Enzyme HSD3B1 (scAT und Leber) und HSD17B12 (scAT), was darauf hindeutet, dass sich die Paritätszahl (bzw. Alter) unterschiedlich auf die Enzymexpression und die Steroidspiegel auswirkt.

Die erhöhte postpartale Glukokortikoid Konzentration im scAT der NBCS im Vergleich zu HBCS-Kühen könnte entweder die lokale Adipogenese durch die Differenzierung von Präadipozyten in reife Adipozyten beeinflussen oder am akuten Entzündungsgeschehen beteiligt sein – oder auch beide Mechanismen beeinflussen.

Die Ergebnisse unserer Beobachtungen tragen zu einem besseren Verständnis der komplexen Regulation des peripartalen Steroidstoffwechsels und der lokalen Steroidumwandlung im AT von Milchkühen bei. Kühe, die beim Kalben überkonditioniert waren, wiesen während der frühen Laktationsperiode eine höhere metabolische Belastung auf, welche durch eine intensivere Körperfettmobilisierung verursacht wurde und mit erhöhten Blutkonzentrationen an Stoffwechselmetaboliten und -hormonen im Vergleich zu normal konditionierten Kühen verbunden war.

Darüber hinaus zeigten überkonditionierte im Vergleich zu normal konditionierten Kühen ein verändertes Steroidprofil im AT sowie Unterschiede in der Abundanz steroidogener Enzyme, welches zusätzlich durch die Parität der Kühe beeinflusst wurde. Weitere Publikationen, abgeleitet aus dem hier etablierten Versuchsaufbau, vertieften das Verständnis über die pathophysiologischen Auswirkungen einer Überkonditionierung auf molekularer Basis (metabolischer Fußabdruck). Um hingegen den ganzheitlichen Steroidstoffwechsels in der Milchkuh darzustellen, bedarf es weitere Untersuchungen unter Einbeziehung zusätzlicher Faktoren, die zur lokalen Steroidbildung beitragen (z. B. weitere steroidogene Enzyme, Steroidrezeptoren, Steroidvorläufer, sulfonierte Steroide). Außerdem könnten Körperflüssigkeiten wie Urin und Milch, aber auch Fäzes sowie zusätzliche Zielgewebe (z. B. verschiedene AT Lokationen, Eierstöcke) in weitere Untersuchungen einbezogen werden.

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## 10 Danksagung

Mein besonderer Dank gilt Frau Prof. Dr. Dr. Helga Sauerwein, die mir die Möglichkeit gab als externe Doktorandin an ihrem Institut mit dieser überaus interessanten Thematik zu promovieren. Ich danke Ihnen für Ihre fortwährende Unterstützung, Förderung und Erreichbarkeit aus der Ferne und für Ihr Vertrauen und die Möglichkeiten, die Sie mir während meiner Doktorandenzeit geboten haben; insbesondere die Kurzzeitstudien an der Università degli Studi di Milano und an der University of Zagreb haben mich auf persönlicher und wissenschaftlicher Ebene bereichert . Herzlichen Dank!

Für die interessanten und förderlichen Statusgespräche sowie für die Übernahme des Koreferats möchte ich herzlich Herrn Prof. Dr. Karl-Heinz Südekum danken.

Auch bedanke ich mich bei Frau Dr. Susanne Häussler für die Übernahme der Zweitbetreuung. Susanne, für deine professionelle Unterstützung und deine Geduld während der Bearbeitung des zweiten Manuskripts danke ich dir sehr.

Herr Prof. Hassan Sadri, Inga Hofs und Isabella Israel möchte ich mich ganz besonders für die fortwährende Hilfe und Unterstützung in der PCR-Analytik während meiner „Stations"-Zeit an der Universität in Bonn bedanken. *Hassan, thank you very much for your support and constructive discussions especially during drafting the first manuscript.* Natürlich sei an dieser Stelle Dr. Morteza Hosseini-Ghaffari, Dr. Christiane Urh, Dr. Laura Webb, Thomas Blees und Barbara Heitkönig für ihre Mitarbeit und Unterstützung in diesem umfangreichen Projekt gedankt.

Vielen Dank an die Mitarbeiter des Hofguts Neumühle, welche mich während meines Versuchs unterstützt haben – insbesondere an Herrn Dr. Christian Koch und Herrn Klaus Korst für die Bereitstellung der Tiere. Lieber Christian, vielen Dank für die zahlreichen interessanten Gespräche und deine Unterstützung; deine unermüdliche Begeisterung für die Wissenschaft war immer wieder inspirierend.

Nicht zu vergessen sind die lieben und geduldigen Kühe, ohne die dieses Projekt nicht möglich gewesen wäre: Leoni, Kanada, Dinkel, Stuttgart, Hoyasver, Ester, Ekrath, Paula, Omeaga, Haifa, Kartago, Gerau, Harfe, Ulme, Hildegard, Silvia, Halma, Lena, Hanni, Himmel, Diva, Mia, Rom, Heda, Drache, Ente, Anita, Mara, Hantel, Gin, Antallia, Kundus, Nelly, Lahn, Pille, Hantel 2, Ella, Lotto.

Herzlichen Dank an Herrn Prof. Dr. Georg Dusel von der Technischen Hochschule in Bingen. Lieber Georg, ohne dich wäre ich niemals auf die Idee gekommen zu promovieren! Schon während meiner Bachelorzeit hast du mein Potenzial erkannt und mich bis heute unterstützt, gefördert, und motiviert. Danke, dass du immer an mich glaubst und für die unzähligen Möglichkeiten die du mir über die ganzen letzten Jahre geboten hast (Besuche von internationalen Fachtagungen, Betreuung von Projekten, Trainee an der University of Manitoba, u.v.m.), welche mich insbesondere in meinem wissenschaftlichen Werdegang weitergebracht haben!

Meiner „persönlichen“ Tierärztin und Freundin Frau Dr. Dörte Frieten gilt für die umfangreichen Probenentnahmen (Biopsien) während des Projekts ein ganz besonderer Dank! Dörte, ich bewundere deinen fortwährenden Respekt und die Liebe zu den Tieren Vielen Dank für deinen fachlichen Input und deine unermüdliche Unterstützung während meines Versuchs.

Für die finanzielle Förderung während meiner Doktorandenzeit möchte ich mich herzlich bei der H. Wilhelm Schaumann-Stiftung bedanken.

Schlussendlich möchte ich mich bei Samson für seine Geduld, Liebe und unermüdliche Unterstützung und Motivation bedanken. Danke, dass du immer an mich glaubst und es ist schön dich und Emil an meiner Seite zu wissen.

## 11 Publications and proceedings derived from this doctorate thesis

***Publications (peer reviewed)***

1. ***Schuh, K.;*** *Häussler, S.; Sadri, H.; Prehn, C.; Lintelmann, J.; Adamski, J.; Koch, C.; Frieten, D.; Ghaffari, M.H.; Dusel, G.; Sauerwein, H. (2021): Blood and adipose tissue steroid metabolomics and mRNA expression of steroidogenic enzymes in periparturient dairy cows differing in body condition. Submitted and under review.*
2. Webb, L. A.; Ghaffari, M. H.; Sadri, H.; **Schuh, K.**; Zamarian, V.; Koch, C.; Trakooljul, N.; Wimmers, K.; Lecchi, C.; Ceciliani, F.; Sauerwein, H. (2020b): Profiling of circulating microRNA and pathway analysis in normal- versus over-conditioned dairy cows during the dry period and early lactation. In Journal of Dairy Science. DOI: 10.3168/jds.2020-18283.
3. Ghaffari, M. H.; Jahanbekam, A.; Post, C.; Sadri, H.; **Schuh, K.**; Koch, C.; Sauerwein, H. (2020a): Discovery of different metabotypes in overconditioned dairy cows by means of machine learning. In Journal of Dairy Science. DOI: 10.3168/jds.2020-18661.
4. Sadri, H.; Ghaffari, M. H.; **Schuh, K.**; Dusel, G.; Koch, C.; Prehn, C.; Adamski, J.; Sauerwein, H. (2020): Metabolome profiling in skeletal muscle to characterize metabolic alterations in over-conditioned cows during the periparturient period. In Journal of Dairy Science 103 (4), pp. 3730–3744. DOI: 10.3168/jds.2019-17566.
5. Ghaffari, M. H.; **Schuh, K.**; Kuleš, J.; Guillemin, N.; Horvatić, A.; Mrljak, V.; Eckersall, P. D.; Dusel, G.; Koch, C.; Sadri, H.; Sauerwein, H. (2020b): Plasma proteomic profiling and pathway analysis of normal and overconditioned dairy cows during the transition from late pregnancy to early lactation. In Journal of Dairy Science 103 (5), pp. 4806–4821.
6. Ghaffari, M. H.; Sadri, H.; **Schuh, K.**; Dusel, G.; Prehn, C.; Adamski, J.; Koch, C.; Sauerwein, H. (2020c): Alterations of the acylcarnitine profiles in blood serum and in muscle from periparturient cows with normal or elevated body condition. In Journal of Dairy Science 103 (5), pp. 4777–4794.
7. Webb, L. A.; Sadri, H.; **Schuh, K**.; Egert, S.; Stehle, P.; Meyer, I.; Koch, C.; Dusel, G.; Sauerwein, H. (2020a): Branched-chain amino acids: Abundance of their transporters and metabolizing enzymes in adipose tissue, skeletal muscle, and liver of dairy cows at high or normal body condition. In Journal of Dairy Science 103 (3), pp. 2847–2863.
8. Ghaffari, M. H.; Sadri, H.; **Schuh, K.**; Dusel, G.; Frieten, Dörte; Koch, C.; Prehn, C.; Adamski, J.; Sauerwein, H. (2019a): Biogenic amines: Concentrations in serum and skeletal muscle from late pregnancy until early lactation in dairy cows with high versus normal body condition score. In Journal of Dairy Science 102 (7), pp. 6571–6586.
9. Ghaffari, M. H.; **Schuh, K.**; Dusel, G.; Frieten, D.; Koch, C.; Prehn, C.; Adamski, J.; Sauerwein, H.; Sadri, H. (2019b): Mammalian target of rapamycin signaling and ubiquitin-proteasome-related gene expression in skeletal muscle of dairy cows with high or normal body condition score around calving. In Journal of Dairy Science 102 (12), pp. 11544–11560.
10. Ghaffari, M. H.; Jahanbekam, A.; Sadri, H.; **Schuh, K.**; Dusel, G.; Prehn, C.; Adamski, J.; Koch, C.; Sauerwein, H. (2019c): Metabolomics meets machine learning: Longitudinal metabolite profiling in serum of normal versus overconditioned cows and pathway analysis. In Journal of Dairy Science 102 (12), pp. 11561–11585.
11. **Schuh, K**.; Sadri, H.; Häussler, S.; Webb, L. A.; Urh, C.; Wagner, M.; Koch, C.; Frahm, J.; Dänicke, S.; Dusel, G.; Sauerwein, H. (2019): Comparison of performance and metabolism from late pregnancy to early lactation in dairy cows with elevated v. normal body condition at dry-off. In Animal: an international journal of animal bioscience, pp. 1–11. DOI: 10.1017/S1751731118003385.

***Proceedings***

1. **K. Schuh**, S. Häussler, G. Dusel, C. Koch, C. Prehn, J. Adamski, H. Sadri, H. Sauerwein (2019): Characterization of the time course of 17 different steroids in serum from cows with elevated versus normal body condition score. 70th Annual Meeting of the European Federation of Animal Science. EAAP, Ghent, Belgium, 26 - 30 August 2019.
2. Ghaffari, M.H.; Jahanbekam, A.; Sadri, H.; Schuh, K.; Prehn, C.; Adamski, J.; Koch, C.; Sauerwein, H. (2019): Machine learning approach reveals a metabolic signature of over-conditioned cows. 70th Annual Meeting of the European Federation of Animal Science. EAAP, Ghent, Belgium, 26 - 30 August 2019.
3. Ghaffari, M.H.; Alaedin, M.T.; **Schuh, K.**; Hofs, I.; Frieten, D.; Koch, C.; Sadri, H.; Sauerwein, H. (2019): Effect of over-conditioning around calving on carnitine palmitoyltransferase gene expression in the liver and skeletal muscle of dairy cows. 17th Intl. Conference on Production Diseases in Farm Animals, ICPD, Bern, Switzerland, June 2019.
4. Ghaffari, M.H.; Alaedin, M.T.; **Schuh, K.**; Sadri, H.; Koch, C.; Frieten, D.; Sauerwein, H. (2019): Effect of over-conditioning around calving on the mRNA abundance of genes related to the mitochondrial protein import machinery in the liver of dairy cows. 17th Intl. Conference on Production Diseases in Farm Animals, ICPD, Bern, Switzerland, June 2019.
5. Ghaffari, M.H.; **Schuh, K.**; Kuleš, J.; Guillemin, N.; Horvatić, A.; Mrljak, V.; Eckersall, P.D.; Dusel, G.; Koch, C.; Sadri, H.; Sauerwein, H. (2019): Characterization of the plasma proteome in normal and overconditioned dairy cows during the transition from late pregnancy to early lactation. XIII. Annual Congress of the European Proteomics Association. Potsdam, Germany, March 2019.
6. Laura A. Webb, H. Sadri, **K. Schuh**, S. Egert, P. Stehle, C. Koch, G. Dusel, H. Sauerwein (2019): Plasma branched-chain amino acids (BCAA) and mRNA abundance of 3 different BCAA transporters in adipose tissue, muscle and liver of dairy cows with high or normal body condition score. Annual Meeting of the American Dairy Science Association, ADSA, Cincinnati, Ohio, July 2019.
7. C. Urh, **K. Schuh**, V. Zamarian, L. Webb, C. Lecchi, M. Alaedin, H. Sadri, M. Ghaffari, G. Dusel, C. Koch, N. Trakooljul, K. Wimmers, F. Ceciliani, H. Sauerwein (2018): Profiling peripheral microRNA in normal- versus over-conditioned dairy cows during dry-off and early lactation. Journal of Animal Science 96 (3), pp. 357-357.
8. Ghaffari, M.H.; Sadri, H.; **Schuh, K.**; Dusel, G.; Koch, C.; Sauerwein, H. (2018): Effect of over-conditioning on mTOR and ubiquitin proteasome gene expression in muscle of dairy cows. 69th Annual Meeting of the European Federation of Animal Science, EAAP, Dubrovnik, Croatia, August 2018.
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Lehr und Versuchsanstalt Hofgut Neumühle 2015