# Mammalian target of rapamycin (mTOR) signaling and ubiquitin-proteasome–(UPS) related gene expression in skeletal muscle of dairy cows with high or normal BCS around calving

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**Interpretive Summary: Mammalian target of rapamycin (mTOR) signaling and ubiquitin-proteasome–(UPS) related gene expression in skeletal muscle of dairy cows with high or normal BCS around calving**. *By Ghaffari et al.,* The regulation of muscle protein turnover during a period of negative nutrient balance needs to be better understood. The objective of this study was to elucidate the effect of body condition around calving on the mRNA abundance of critical components of the mTOR pathway and UPS in the skeletal muscle (M. semitendinosus) of dairy cows. The findings from the current study revealed that over-conditioning around calving might stimulate muscle protein turnover in early lactation, as indicated by the changes in the mRNA abundance of key components of mTOR signaling and the UPS.



RUNNING HEAD: mTOR AND UPS SIGNALING IN PERIPARTURIENT COWS

# Mammalian target of rapamycin (mTOR) signaling and ubiquitin-proteasome–(UPS) related gene expression in skeletal muscle of dairy cows with high or normal BCS around calving

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## ABSTRACT

The objective of the current study was to investigate the effects of over-conditioning around calving on gene expression of key components of mammalian target of rapamycin (mTOR) pathway and ubiquitin-proteasome system (UPS) in skeletal muscle as well as the AA profiles in both serum and muscle of periparturient cows. Fifteen weeks ante partum, 38 multiparous Holstein cows were allocated to either a high or a normal body condition group (**HBCS** and **NBCS**, each n = 19) and were fed different diets until dry-off (d -49 relative to calving) to amplify the difference. The groups were also stratified for comparable milk yields (NBCS: 10,361 ± 302 kg; HBCS: 10,315 ± 437 kg). At dry-off the cows in the NBCS group (parity: 2.42 ± 1.84, body weight: 665 ± 64 kg) had a BCS < 3.5 and backfat thickness (BFT) < 1.2 cm, whereas the HBCS cows (3.37 ± 1.67; body weight: 720 ± 57 kg) had BCS > 3.75 and BFT > 1.4 cm, respectively. During the dry period and the subsequent lactation, both groups were fed identical diets but maintained the BCS and BFT differences. Blood samples and skeletal muscle biopsies (M. semitendinosus) were repeatedly (d -49, +3, +21, and +84 relative to calving) collected for assessing the concentrations of free AA and the mRNA abundance of various components of mTOR and UPS. The differences in BCS and BFT were maintained throughout the study. The circulating concentrations of most AA with the exception of Gly, Gln, Met, and Phe increased in early lactation in both groups. The serum concentrations of Ala (d +21 and +84) and Orn (d +84) were lower but those of Gly, His, Leu, Val, Lys, Met, Orn on d -49 and Ile on d +21 were greater in HBCS than in NBCS. The serum concentrations of 3-methylhistidine (3-MH), creatinine, and 3-MH/creatinine increased after calving (d +3) but did not differ between the groups. The muscle concentrations of all AA (except for Cys) remained unchanged over time and did not differ between groups. The muscle concentrations of Cys were greater on d -49, but tended to be lower on d +21 in

HBCS than in NBCS cows. On d +21, *mTOR* and *eukaryotic translation initiation factor 4E binding protein 1* mRNA abundance was greater in HBCS than in NBCS cows, whereas *ribosomal protein S6 kinase 1* was not different between the groups. The mRNA abundance of *ubiquitin-activating* enzyme *1* (d +21), *ubiquitin-conjugating enzyme 1* (d +21), *atrogin-1* (d +21), and *ring finger protein-1* (d +3) enzymes were greater in HBCS than in NBCS; while *ubiquitin-conjugating enzyme 2* was not different between the groups. The increased mRNA abundance of key components of *mTOR* signaling and of muscle-specific ligases of HBCS cows may indicate a simultaneous activation of anabolic and catabolic processes, and thus increased muscle protein turnover, likely as a part of the adaptive response to prevent excessive loss of skeletal muscle mass during early lactation.

**Key words:** mammalian target of rapamycin, ubiquitin-proteasome system, body condition score, transition cows

## INTRODUCTION

In dairy cows, the transition from late gestation to early lactation is associated with extensive changes in metabolic, endocrine, and immune functions (Drackley, 1999). In early lactation, dairy cows typically experience a negative energy balance, because insufficient feed intake cannot support the increased nutrient demand for milk synthesis at the onset of lactation. Besides fat (De Vries and Veerkamp, 2000), mobilization of body protein reserves is also necessary to provide amino acids (AA) for (milk) protein synthesis, direct oxidation, or gluconeogenesis (Plaizier et al., 2000; Kuhla et al., 2011; Sadri et al., 2016). In early lactation, reduced skeletal muscle protein mass, as well as decreased diameter of M. longissimus dorsi, were demonstrated in dairy cows at

the early stage of lactation in comparison with late stages (Phillips et al., 2003; Kessel et al., 2008). Body condition can influence the extent of protein mobilization in dairy cows at parturition and in early lactation. Cows that are over-conditioned around calving have a more severely depressed feed intake postpartum (p.p.), leading to a more pronounced negative energy balance (NEB) and subsequently they are more prone to develop a variety of metabolic abnormalities than moderately conditioned (NBCS) cows (Holtenius et al., 2003; Roche et al., 2009). Pires et al. (2013) reported that cows with low body condition (BCS $\leq$ 2.5) mobilized less body fat but had a more intense muscle protein catabolism during the first weeks of lactation compared with over-conditioned cows (HBCS $\geq$ 3.75). The mechanisms underlying peripartum protein mobilization may be mediated by the endocrine changes including hypoinsulinemia and diminished muscle responsiveness to insulin, growth hormone, and insulin-like growth factor (IGF-1) concentrations (Bell et al., 2000) and the up-regulated expression of components of proteolytic pathways in skeletal muscle (Chibisa et al., 2008). Cellular and molecular events, including the components involved in protein turnover signaling pathways, support the physiological and metabolic adaptations during the time of NEB when body fat and protein reserves are utilized to support lactation. Recent reports have demonstrated that the mammalian target of rapamycin (mTOR) pathway and the ubiquitin-proteasome system (UPS) were influenced in protein turnover of muscle and other body tissue in dairy cows (Castro et al., 2016; Sadri et al., 2016; Dong et al., 2017).

The loss of body protein in early lactation can range from 12 to 21 kg within the first 5 wk p.p. (Komaragiri and Erdman, 1997; Komaragiri et al., 1998; Chibisa et al., 2008). There are two distinct multi-protein complexes (mTORC1 and mTORC2) that integrate multiple signals of satiety from AA abundance, hormones, and growth factors (Sheldon et al., 2017). The mTORC1 complex controls protein synthesis, cellular proliferation, cell size, and gene expression, whereas the

mTORC2 complex regulates cytoskeleton formation in response to nutrients and growth factors (Jacinto et al., 2004). For protein degradation, the UPS is regarded as the central proteolytic pathway in the muscle (Rock et al., 1994). It requires the coordinated reactions of 3 enzymes including E1 (an ubiquitin-activating enzyme), E2 (an ubiquitin-conjugating enzyme), and E3 (ubiquitin ligases) (Schulman and Harper, 2009; Yael et al., 2010). The two muscle-specific E3 ubiquitin ligases (*atrogin*-1 and *MuRF*-1) are specific markers of muscle wasting and are upregulated during muscle-wasting conditions (Franch and Price, 2005; Foletta et al., 2011). Greenwood et al. (2009) observed a 2.1-fold greater upregulation of ubiquitin mRNA abundance in skeletal muscle of Holstein dairy cows after calving compared with the prepartum stage, indicating increased proteolytic activity. However, information on the mobilization of body reserves in HBCS versus NBCS cows is limited, in particular for what concerns the regulation of protein metabolism in the skeletal muscle of cows during late gestation and early lactation when comprehensive endocrine and metabolic changes occur.

To address these issues, we used an experimental model in dairy cows for high versus normal mobilization around calving by feeding different diets (energy levels) before dry-off and based on pre-selection [as quantified by body condition score (BCS) and backfat thickness (BFT)] in the previous and the ongoing lactation (Schuh et al., 2019). We hypothesized that cows calving with high BCS are metabolically challenged during early lactation due to a more severe NEB and intense mobilization of body fat that may also affect the regulation of specific signaling components related to protein synthesis and protein degradation. Therefore, we evaluated the effects of BCS around calving on mRNA abundance of key factors of the mTOR pathway and UPS in the skeletal muscle of dairy cows.

## MATERIALS AND METHODS

### *Animals, Management, and Treatments*

The experiment was conducted at 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 German Animal Welfare Act and were approved by the local authority for animal welfare affairs [Landesuntersuchungsamt Rheinland-Pfalz, (G 14-20-071)] Koblenz, Germany. The basic set-up of the trial with the performance results as well as the data of "classical" variables, i.e. NEFA, BHB, insulin, glucose, leptin, IGF-1, oxidative and thyroid hormone status, assessed in blood serum were already described by Schuh et al. (2019). In brief, 38 multiparous German Holstein cows (average parity: 2.9 ± 0.30, mean ± SEM) were allocated 15 wk before their anticipated calving date to either **NBCS** (n = 19) or **HBCS** (n = 19) group. These two groups were fed differently (for the diets see Table 1) as detailed below from wk 15 to 7 before the anticipated calving date to reach different targets for BCS and back fat thickness (BFT) at dry-off (NBCS: <3.5 and <1.2 cm; HBCS: >3.75 and >1.4 cm). The two groups were initially pre-selected from the entire herd (150 heads) by their history of BCS and BFT records from the year preceding the trial to find cows divergent in both variables to have two groups with equal numbers. The preselected cows were also stratified for comparable milk yields (NBCS: 10,361 kg ± 302 kg; HBCS: 10,315 ± 437 kg). From week 15 to 7 before the anticipated calving date, NBCS cows were fed a low-energy ration [6.8 $NE_L$ (MJ/kg of DM)], while HBCS cows were fed a high-energy ration [7.2 $NE_L$ (MJ/kg of DM)]. During the dry period and subsequent lactation, both groups received the identical diet. All diets were fed as total mixed ration (TMR) consisting of 63% roughage and 37% concentrate in the high-energy diet, or 74% roughage and 26% concentrate in the low-energy diet. The diets were balanced to meet or exceed the nutritional requirements of Holstein cows according

157 to the recommendation of the Society of Nutrition Physiology in Germany (GfE, 2001). One person
158 was monitoring both BCS and BFT every two wk during the entire period of the trial (15 wk a.p. to
159 12 wk p.p.). The BCS was estimated on a 5-point scale (Edmonson et al., 1989), while BFT was
160 assessed in the sacral region using ultra-sonography (AGROSCAN L, ALR 500, 5 MHz, linear-
161 array transducer, Echo Control Medical, Angoulême, France). Net energy balance (EB) was
162 calculated from week 3 a.p. until week 12 p.p. as previously described (Schuh et al., 2019).

163 ***Sampling and Laboratory Analyses***

164 Individual daily feed intake was recorded from week 3 a.p. until week 12 p.p. as previously
165 described (Schuh et al., 2019). Total mixed rations (TMR), as well as the concentrate feed, were
166 sampled every 2 weeks and stored at -20 °C until analysis. Dry matter of diets was determined by
167 drying at 60 °C for 24 h and then at 105 °C for 3 h. The nutrient composition of the feed samples
168 was carried out according to the methods of the Association of German Agricultural Analytic and
169 Research Institutes (Naumann and Bassler, 2004). Samples were analyzed for CP, utilizable CP,
170 crude ash, crude fat, crude fiber, NDF, ADF, and NFC. The minerals (Ca, P, Mn, Na, and K) were
171 analyzed by x-ray fluorescence analysis. The energy content (ME and NEL) of the diet was
172 calculated according to the German Society of Nutrition Physiology (GfE, 2009).

173 Blood samples were collected from the *Vena caudalis mediana* before the morning feeding
174 on d -49, 3, 21, and 84 relative to calving. After clotting and subsequent centrifugation (10 min,
175 2,000 × *g*), the serum was obtained and stored at -20 °C until analysis. The blood samples collected
176 for metabolomics were stored at -80 °C until analysis. Biopsies from M. semitendinosus were
177 collected on the same days of blood sampling. The animals were sedated by intravenous injection of
178 Xylazine (20 mg/mL, 0.1 mL/100 kg BW; CP-Pharma Handels GmbH, Burgdorf, Germany) and
179 fixed in a headlock. The biopsy area was cleaned, shaved, and disinfected with 70% isopropyl

alcohol. Muscle samples were obtained under local anesthesia with procaine hydrochloride (20 mg/mL, 8 mL per biopsy; Richter Pharma AG, Wels, Austria) by a 12 G × 20 cm Core Tissue Biopsy Needle with a Bard Magnum® biopsy instrument (Bard Inc., Tempe, AZ). After tissue extraction, oxytetracycline hydrochloride was applied on the skin (25 mg/mL, EngemycinTM, MSD Animal Health Innovation GmbH, Schwabenheim an der Selz, Germany) and a ketoprofen injection (100 mg/mL, 3 mL/100 kg BW; Streuli Pharma AG, Uznach, Germany) was given to prevent infection and pain. Tissue samples were immediately snap-frozen in liquid nitrogen and stored at −80 °C until analysis.

Serum 3-methylhistidine (3-MH) was analyzed by via high performance liquid chromatography (HPLC) in a RF-10A XL fluorescence detector (Shimadzu, Kyoto, Japan) based on o-phtaldialdehyde/3-mercaptopropionic acid derivatization as previously described (Fürst et al., 1990).

The AA profiles in serum and skeletal muscle, as well as serum creatinine, were determined by liquid chromatography-electrospray ionization-tandem mass spectrometry (LC-ESI-MS/MS) profiling through targeted metabolomics using the Absolute*IDQ*™ p180 Kit (Biocrates Life Sciences AG, Innsbruck, Austria). This kit was validated according to the European Medicines Agency guidelines (EMEA Quality guidelines), which implies a proof of reproducibility within a given error range. All analyses were performed in the Helmholtz Zentrum München (GmbH), German Research Center for Environmental Health, Genome Analysis Center. In the case of serum, 10 µL of the thawed sample was applied directly to the assay. In case of muscle, frozen samples were homogenized and extracted using homogenization tubes with ceramic beads (1.4 mm) and a Precellys 24 homogenizer with an integrated cooling unit (PEQLAB Biotechnology GmbH, Erlangen, Germany). Three µL of dry ice cooled mixture of ethanol/phosphate buffer (85/15 v/v)

were added to each mg of frozen muscle tissue. After centrifugation, 10 µL of the homogenate supernatant were applied to the well plate of the p180 kit. The assay procedures of the Absolute*IDQ*™ p180 Kit, the detailed description of the tissue preparation and the metabolite nomenclature were described in details elsewhere (Zukunft et al., 2013 and 2018). Sample handling was performed by a Hamilton Microlab STAR™ robot (Hamilton Bonaduz AG, Bonaduz, Switzerland) and an Ultravap nitrogen evaporator (Porvair Sciences, Leatherhead, U.K.), beside standard laboratory equipment. Mass spectrometric analyses were done on an API 4000 triple quadrupole system (Sciex Deutschland GmbH, Darmstadt, Germany) equipped with a 1200 Series HPLC (Agilent Technologies Deutschland GmbH, Böblingen, Germany) and an HTC PAL autosampler (CTC Analytics, Zwingen, Switzerland) controlled by the software Analyst 1.6.1. Data evaluation for quantification of metabolite concentrations and quality assessment were performed with the Met*IDQ*™ software package, which is an integral part of the Absolute*IDQ*™ Kit. Internal standards were used as a reference for the calculation of metabolite concentrations. The concentrations of the serum samples are given in µmol/L, the concentrations of the tissue samples in pmol/mg tissue.

Total RNA was extracted using the Qiagen reagent (Qiagen, Hilden, Germany) from the muscle homogenates according to the manufacturer's protocol. The extracted RNA was purified using the RNeasy® Mini Kit (Qiagen) including the On-Column DNase I treatment to remove residual genomic DNA from the RNA samples. The quantity and purity of RNA were evaluated by measuring the absorbance at 260 nm and 280 nm by the Nanodrop 1000 spectrophotometer (PEQLab Biotechnology). The RNA integrity was assessed using ethidium bromide-denaturing RNA electrophoresis and re-checked in randomly selected samples using an Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany) with the RNA 6000 Nano Kit system

according to the manufacturer's protocol to determine RNA integrity number (RIN = 7.63 ± 0.17 SD). Only samples with a 28s/18s ratio ≥ 2.0 and RNA integrity number ≥ 7.0 were used for downstream applications. The reverse transcription was conducted with 250 ng of total RNA per 20 µL reaction using RevertAid™ Reverse Transcriptase (200 U/µL), 20 U of RiboLock ribonuclease inhibitor (Fermentas, St. Leon-Rot, Germany), and 500 µM of each deoxynucleotide triphosphate, with 200 pmol of random hexamer primers (Sigma-Aldrich, Nümbrecht, Germany) for 10 min at 27 °C, 60 min at 42 °C, and 1 min at 99 °C. Each run included a no-reverse-transcriptase (-RT) control and a no-template control (NTC). The reverse transcription was performed in duplicate for each sample. The analyses by quantitative real-time PCR (qPCR) were performed using a Mx3000P PCR cycler (Stratagene, Amsterdam, The Netherlands, and Agilent, Santa Clara, CA) following by the original Minimum Information for Publication of qPCR Experiments (MIQE) guidelines (Bustin et al., 2009). The qPCR conditions and the characteristics of the primers are presented in Table 2. The reaction was performed in triplicate in a total volume of 10 µL composed of 2 µL cDNA (diluted 1:4) as a template, 1 µL of the assay-specific primer mix, 5 µL of the SYBR Green JumpStart Taq Readymix (Sigma–Aldrich Chemie GmbH, Steinheim, Germany), and 2 µL water. An NTC for quantitative PCR, as well as an NTC and -RT of cDNA, were included in each run. For each PCR reaction, a standard curve using a serial dilution of cDNA was generated to calculate efficiency-corrected relative quantities of the targets [run-specific target amplification efficiency].

To determine the most stably expressed genes across treatments for subsequent data normalization, a set of 5 genes (Saremi et al., 2012) was tested, and their stability was evaluated using qBASE$^{plus}$ version 2.0 (Biogazelle, Ghent, Belgium). Three reference genes including low-density lipoprotein receptor-related protein 10 (*LRP10*), RNA polymerase II (*POLR2A*), and emerin (*EMD*) were determined as the most stable reference genes (Table 2). All subsequent calculations

and data quality controls were performed using qBASE+ software (Hellemans et al., 2007). The output data from the software were calibrated normalized relative quantities (CNRQ values).

### *Statistical Analyses*

A repeated-measures model was fitted to data using the PROC MIXED procedure of SAS (version 9.4; SAS Institute Inc., Cary, NC). Before analysis, all data were tested for normality of distribution by evaluating the Shapiro–Wilk statistic using the UNIVARIATE procedure of SAS and where appropriate, they were transformed using a log10 transformation. The model consisted of treatment, time (day relative to calving), and interaction of treatment and time as fixed effects, and cow as the random effect. An autoregressive (order 1) covariance structure was chosen based on the Akaike and Bayesian information criteria. The final results of the mRNA abundance were calculated by qBASE+ (i.e., the calibrated normalized relative quantities values were used for statistical analysis of the mRNA data). When differences were detected in the treatment or interaction, means separation was conducted using a Tukey's adjustment for the probability. The threshold of significance was set at $P \leq 0.05$; trends were declared at $0.05 < P \leq 0.10$.

## RESULTS

### *Body Condition and Animal Performance*

A more detailed description of variables characterizing body condition as well as animal performance was reported previously (Schuh et al., 2019). In brief, BCS, BFT, and BW (Supplemental Figure S1) were greater in HBCS than in NBCS cows at enrolment (15 wk a.p.). However, when both groups received the same diets during the dry period, they increased their body condition whereby the previously established differences ($\Delta$ = 0.7 BCS points and 1.1 cm

BFT) were largely maintained until the week before calving (Figure 1A, B). Body condition declined during lactation in both groups, but the losses were bigger in HBCS than in NBCS cows (Figure 1C, D). Dry matter intake (Figure 1E) was greater in NBCS than in HBCS cows until calving when both groups reached the same nadir 1 wk p.p. During the subsequent weeks, NBCS cows had a faster increase in feed intake; the difference between groups leveled off in wk 11 p.p. The calculated EB (Figure 1F) was greater in NBCS than in HBCS cows during the a.p. period and also reached positive values about 2 wk earlier than in the HBCS cows.

***Concentrations of AA in Serum and Skeletal Muscle***

The concentrations of serum AA during the transition from late pregnancy to early lactation are presented in Figure 2. Serum Arg, Asn, Asp, Glu, Ser, and Thr were not different between the groups. Circulating concentrations of total AA and most AA except Gly, Gln, Met, and Phe increased with time of lactation in both groups. The serum concentrations of Ala ($P < 0.01$, d +21 and +84) and Orn ($P = 0.01$, d +84) were lower but those of Gly (d -49), His (d -49), Leu (d -49), Val (d -49), Lys (d -49), Ile (d +21), Met (d -49), and Orn (d -49) were greater ($P < 0.05$) in HBCS than in NBCS cows. The serum concentrations of Gln ($P = 0.07$, d -49) and Tyr ($P = 0.09$, d -49) tended to be greater in HBCS than in NBCS cows, while those of Cys, Phe, Pro, and Trp were similar between groups. The serum concentrations of total free AA were greater ($P < 0.01$) in HBCS than in NBCS cows on d -49 and +21. The serum concentrations of 3-MH, creatinine, and 3-MH/creatinine were elevated on d +3 ($P < 0.01$) as compared with other time-points, but did not differ between groups (Figure 3).

The concentrations of muscle AA are presented in Table 3. The concentrations of all AA except for Cys and His remained unchanged during the observation period and did not differ between

294 groups. The muscle concentrations of Cys were greater ($P < 0.05$) in HBCS than in NBCS cows on
295 d -49; but tended ($P = 0.07$) to be lower on d +21. For time-related differences, the muscle
296 concentrations of His ($P = 0.03$) increased from late pregnancy to early lactation in both groups.
297 The concentrations of total free AA in muscle were neither affected by group nor by time, and there
298 was no group × time interaction.
299
300 ***mRNA Abundance in the Skeletal Muscle***
301 As shown in Figure 4, the mRNA abundance of *mTOR* was greater ($P < 0.05$) and that of
302 eukaryotic translation initiation factor 4E binding protein 1 (*4E-BP1*) tended ($P = 0.07$) to be
303 greater in HBCS than in NBCS cows on d +21, but there was no time or group × time interaction.
304 The mRNA abundance of ribosomal protein S6 kinase 1 (*S6K1*) was neither affected by group nor
305 by time, and there was no group × time interaction (Figure 4).
306 The mRNA abundance of ubiquitin-activating enzyme (*UBA1*), ubiquitin-conjugating enzyme 1
307 (*UBE2G1*), and *atrogin-1* were not affected by group or time (Figure 5). However, a group × time
308 interaction was observed for the mRNA abundance of *UBA1* ($P = 0.001$), *UBE2G1* ($P = 0.04$), and
309 *atrogin-1* ($P = 0.03$): they were greater on d +21 in HBCS than in NBCS cows (Figure 3). The
310 abundance of ubiquitin-conjugating enzyme 2 (*UBE2G2*) mRNA was not affected by group or time,
311 and also a group × time interaction was not observed (Figure 5). The mRNA abundance of muscle
312 ring finger protein-1 (*MuRF-1*) was greater ($P = 0.05$) in HBCS than in NBCS cows on d +3, but
313 there was no time or group × time interaction (Figure 5).
314
315 **DISCUSSION**

316 It has been well documented that high yielding dairy cows may experience negative nutrient
317 balance during the periparturient period (Drackley, 1999; Rukkwamsuk et al., 1999), leading to
318 mobilization of body fat and muscle protein as feed intake alone cannot meet the
319 nutrients requirements (Heuer et al., 1999; Kuhla et al., 2011; van der Drift et al., 2012). High
320 yielding cows experience some degree of oxidative stress in early lactation and cows with higher
321 BCS and greater BCS losses at parturition are more sensitive to oxidative stress compared with low
322 or medium BCS cows (Bernabucci et al., 2005). Previously, Schulz et al. (2014) described the
323 pathophysiological serum alterations such as high values of NEFA, aspartate transaminase, and
324 glutamate dehydrogenase in over-conditioned cows developing subclinical ketosis in early
325 lactation. Mann et al. (2016) have recently reported peripartal skeletal muscle accretion and
326 proteolysis in cows with excess energy consumption compared with cows fed a high fiber,
327 controlled energy diet during the prepartum period. The dry period plane of energy did not lead to
328 changes in BW or BCS (high energy group = 3.1 ± 0.2 and control group = 3.1 ± 0.3; mean ± SE),
329 as reported earlier (Mann et al., 2015). Thus, in the study reported by Mann et al. (2016), peripartal
330 skeletal muscle accretion and proteolysis have been investigated in mid-range-scoring cows and
331 indeed no comparison has been made concerning mobilization of body reserves in over-conditioned
332 compared with normal conditioned cows.

333 During the transition period, rapid fetal growth, lactogenesis, and synthesis of milk protein, as
334 well as gluconeogenesis require large amounts of certain AA that are withdrawn from the
335 circulation and may lead to an imbalanced AA pattern in the blood (Kuhla et al., 2011). Plasma AA
336 status is a net result of all fluxes involved in AA absorption from the digestive tract, muscle protein
337 turnover, liver uptake of glucogenic AA, milk protein synthesis as well as metabolic rate (Maeda et
338 al. 2012; Samman et al., 2014). Regarding the changes in serum AA concentrations in the current

study, over-conditioned cows had higher Gly, His, Val, Leu, Lys, Met, Orn, and Tyr concentrations in the serum than NBCS cows on d -49. The reason for the changes observed in the serum AA concentrations of HBCS and NBCS cows on d -49 is not clear, but might be, at least in part, due to the different diets used in these cows from wk 15 to 7 before calving, as the observed differences largely disappeared on d +3, i.e., after receiving the identical diets. In the current study, the concentrations of Ala, Asn, Glu, Cys, Pro, Val, Leu, Orn, Lys, Thr, Trp, and Tyr for both groups decreased from d -49 to d +3, with nadir concentrations on d +3 and a gradual increase thereafter. These reductions in the serum concentrations might be due to the decreased DMI around calving (Figure 1E) and/or an increase in the uptake of AA into tissues, in particular, the mammary gland (Verbeke et al., 1972). Skeletal muscle, the main labile source of AA in the body, plays important roles in maintaining AA homeostasis. Thus, a shift toward mobilization of body reserves including muscle protein at the onset of lactation as well as increased DMI with time of lactation may be responsible for the return of most serum AA concentrations to their prepartum values within a few wk after calving (Meijer et al., 1995).

Moreover, the lower serum concentrations of Ala in HBCS cows during the p.p. period may be related to a greater demand for gluconeogenic AA for the liver (Chibisa et al., 2008) that is likely coupled with a reduced supply of ruminal propionate to the liver as a result of decreased DMI after calving (Schuh et al., 2019). In support of this, the quantitative data on liver metabolism of AA have shown an increased contribution of Ala to the immediate postpartum increment in the liver release of glucose likely through its role in the inter-organ transfer of nitrogen from catabolized AA (Larsen and Kristensen, 2013). In the current study, the concentrations of most AA (except for Cys and His) in muscle remained fairly constant throughout the observation period despite a decline in serum levels. The reason for these observations is not clear, but muscle appears to have increased

362 transport activity to offset the decline in serum concentrations and thus maintain intracellular
363 concentrations of the respective AA assuming a single time point measurement is reflective of the
364 steady-state.

365 In the current study, the serum 3-MH concentrations, as an indicator of protein breakdown
366 (Harris and Milne, 1981), and the ratio of 3-MH: creatinine (a measure of the fractional catabolic
367 rate of myofibrillar protein) did not differ between groups but were elevated on d +3 in both groups
368 and decreased thereafter. This may reflect an increased muscle protein degradation around
369 parturition since 3-MH stems from the methylation of histidine residues in actin and in myosin
370 (Asatoor and Armstrong, 1967; Hardy and Perry, 1969) and is released into the circulation during
371 muscle degradation. The onset, as well as the duration and extent of protein and energy
372 mobilization from body reserves during the transition period, are highly variable in dairy cows (van
373 der Drift et al., 2012). According to plasma 3-MH concentrations and muscle thickness,
374 mobilization of protein starts ante partum, interestingly even before the onset of lipid mobilization
375 in most cows (van der Drift et al., 2012), and continues to 3 to 5 wk after parturition (Zurek et al.,
376 1995; Doepel et al., 2002; van der Drift et al., 2012). Pires et al. (2013) reported a trend for lower
377 plasma creatinine and a greater 3-MH:creatinine ratio in cows with low BCS (≤ 2.5) as compared
378 with medium (2.75 to 3.5) and high BCS (≥ 3.75) cows, pointing to less muscle mass but more
379 intense mobilization of muscle protein in lean cows. The lack of differences in the plasma
380 creatinine and 3-MH:creatinine ratio of medium and high BCS cows in the study reported by Pires
381 et al. (2013) is in agreement with our observations in the present study. The cellular regulation of
382 skeletal muscle proteolysis in dairy cows in response to negative energy and protein balance is not
383 well described. However, it is well documented that the UPS plays a major role in regulating
384 skeletal muscle proteolysis (Attaix et al., 1994). The proteins targeted for the degradation by the

385 UPS are first tagged with ubiquitin, mediated by the ATP-dependent E1 class of ubiquitin-
386 activating enzymes (Glickman and Ciechanover, 2002; Nandi et al., 2006). Once activated,
387 ubiquitin is transferred to a member of the E2 class of ubiquitin-conjugating enzymes and is, finally
388 conjugated to the target protein with a specific E3 class of ubiquitin ligases. This tagged protein is
389 then proteolyzed by the proteasome enzyme complex (Glickman and Ciechanover, 2002; Nandi et
390 al., 2006). In the current study, the mRNA abundance of *UBA1, UBE2G1,* and *atrogin-1* on d +21
391 were greater in HBCS as compared with NBCS. These findings may indicate upregulation of the
392 UPS system at the mRNA level, which was accompanied by lower DMI and more pronounced
393 NEB. The two major muscle-specific E3 ubiquitin ligases, MuRF-1 and atrogin-1, are specific
394 indicators for the activation of the UPS, and their abundance is crucial for skeletal muscle
395 degradation in a catabolic state (Bodine et al., 2001; Gomes et al., 2012; Foletta et al., 2011).
396 However, our data show that besides *UBA-1* and *UBE2G1*, only *atrogin-1* mRNA was affected by
397 BCS on d +21 being more abundant in HBCS than in NBCS cows. For *MuRF-1*, the mRNA
398 abundance was greater in HBCS on d +3, pointing to the differential response of the two ligases to
399 the metabolic changes occurring during early lactation as influenced by over-conditioning. It seems
400 that the differential expression of these ligases in certain experimental conditions is not uncommon,
401 as reported previously in mice (Frost et al., 2007; Yoshida et al., 2010), neonatal pigs (Suryawan
402 and Davis, 2014), and neonatal calves (Sadri et al., 2017). It is known that the downstream
403 substrates of MuRF-1 and atrogin-1 are not similar and besides degradation, they are also involved
404 in regulating other physiological functions (Foletta et al., 2011). The myogenic transcription factors
405 MyoD and myogenin are known as protein target substrates for atrogin-1, and hence atrogin-1 may
406 play a role in regulating muscle size (Foletta et al., 2011). In contrast to atrogin-1, MuRF-1
407 preferentially interacts with and degrades myofibrillar protein components such as titin (Centner et

al., 2001) and myosin light chain (MLC)1, and MLC2 (Cohen et al., 2009). Furthermore, atrogin-1 has been proposed to regulate the substrate targets and thus affecting muscle protein synthesis and muscle growth, whereas MuRF-1 may play a role in the control of protein degradation and might also contribute to skeletal muscle metabolism.

Interestingly, in the current study, upregulation of *atrogin-1* coincided with the greater abundance of *mTOR* and *4E-BP1* mRNA. The underlying molecular mechanism responsible for the upregulation of key components of *mTOR* (a major regulator of protein synthesis) in HBCS cows on d +21, despite greater *atrogin-1* mRNA abundance, remains to be clarified; however, this might reflect a simultaneous activation of anabolic and catabolic processes resulting in greater protein turnover. Two proteolytic systems, the UPS and lysosomal system, are mainly responsible for the degradation of proteins and organelles, both working in tandem (Ciechanover, 2005). When UPS is stimulated (as reflected by the increase of *atrogin-1* mRNA abundance in the current study), the proteolytic system, regulated by lysosomal degradation is also increased (Lilienbaum, 2013). Interestingly, a physical association has been reported between mTOR and lysosomes that seem to play a critical role in AA mediated mTOR activation (Sancak et al., 2010; Narita and Inoki, 2012). The RAG-regulator complexes in response to increased autophagy, mediate AA-mediated mTOR recruitment to the lysosome surface. As a consequence, the formation of mTOR-autophagy spatial coupling compartment may allow activation of mTOR and autophagy in a mutually reinforcing manner and thus proposing a mechanism for the simultaneous activation of anabolic and catabolic processes (Sancak et al., 2010; Narita and Inoki, 2012). Taken together, over conditioning around calving was associated with the enhanced expression of the two major muscle-specific ligases. As a consequence, proteolysis might have been stimulated and the differential expression of *MuRF-1* and *atrogin*-1 which coincided with the greater mRNA abundance of key components of mTOR

signaling (in case of *atrogin-1*) could be interpreted as an adaptive response of protein metabolism that helps to prevent excessive loss of skeletal muscle mass during early lactation in these cows. As reported previously from this experiment (Schuh et al., 2019), the circulating NEFA concentrations in the HBCS group increased earlier and to greater levels than in the NBCS group, pointing to more intensive body fat mobilization in the HBCS cows. Replenishment of tricarboxylic acid (TCA) cycle intermediates through other metabolites than propionate, such as lactate and gluconeogenic AA become more important when endogenous glucose demand exceeds propionate supply to offset the loss of TCA cycle intermediates from mitochondria over time (Owen et al., 2002; White, 2015). Thus, it is also likely that a greater protein turnover in the muscle of HBCS cows plays a role in supporting glucose and lipid oxidation, a process known as anaplerosis, which seems to more important than its direct contribution to energy supply (Owen et al., 2002; White, 2015). A greater abundance of *BCKDHA* mRNA, participating in the conversion of branched-chain AA (BCAA) to propionyl-CoA, in the subcutaneous adipose tissue of HBCS cows compared with that of NBCS cows (our unpublished data) may further indicate a higher anaplerosis and an increased use of these AA in the peripheral tissues of HBCS cows. In support of this, Schäff et al. (2012) have reported that more intensive body fat mobilization during the postpartum period in dairy cows was associated with a lower DMI and an increased anaplerosis, TCA cycling, and mitochondrial oxidation of acetyl-CoA. Together, our data indicate that an intensive body fat mobilization of HBCS cows during the postpartum period may lead to augmenting nutrient catabolism for anaplerosis and mitochondrial respiration (Schäff et al., 2012), providing a molecular link between muscle protein metabolism and lipid oxidation, known as anaplerosis.

## CONCLUSION

454 Cows calving with high BCS were metabolically challenged during early lactation, associated
455 with the greater mRNA abundance of *MuRF-1* (on d +3) as well as *UBA-1*, *UBE2G1*, and *atrogin-1*
456 (on d +21), which may be related to upregulation of the UPS and, consequently, stimulation of
457 protein degradation in the muscle tissue. The observed upregulation of key components of *mTOR* in
458 HBCS cows on d +21, in spite of the increase in *atrogin-1* mRNA, may point to simultaneous
459 activation of anabolic and catabolic processes, probably serving as an adaptive response of protein
460 metabolism that may prevent excessive loss of skeletal muscle mass during early lactation. Further
461 studies that address changes in body composition, nitrogen balance, and whole-body and skeletal
462 muscle protein turnover, in combination with expression and activity patterns of intracellular
463 regulators of muscle mass, are required to unravel the cellular mechanisms contributing to the
464 regulation of skeletal muscle proteolysis in high- versus normal-conditioned cows.

465

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## Legend of the Figures

**Figure 1.** Changes of body condition score (A), back fat thickness (B), body condition score loss (C), back fat thickness loss (D), dry matter intake (E), and energy balance (F) of normal-conditioned (NBCS) and over-conditioned (HBCS) cows during the experimental period (n = 18 per treatment). Symbols indicate a difference (*$P < 0.05$; **$P < 0.01$) or a trend (†$P < 0.1$) between the groups at a given time. Data for BCS, BFT, BCS loss, BFT loss, DMI, and energy balance are from Schuh et al. (2019). Data are presented as means ± SEM.

**Figure 2.** Serum amino acids concentrations (µmol/L) of normal-conditioned (NBCS) and over-conditioned (HBCS) cows during the observation period (n = 18 per treatment). Symbols indicate a difference (*$P < 0.05$; **$P < 0.01$) or a trend (†$P < 0.1$) between the groups at a given time. Data are presented as means ± SEM.

**Figure 3.** Serum concentrations of 3-Methylhistidine (A, 3-MH), creatinine (B), 3-MH/creatinine (C) of normal-conditioned (NBCS) and over-conditioned (HBCS) cows during the observation period (n = 18 per treatment). Results are presented as means ± SEM. Different capital letters (A, B) indicate differences between time points within HBCS cows; different lowercase letters (a, b) stand for differences between time points within NBCS cows (n = 18 per treatment).

**Figure 4.** The mRNA abundance of genes related to the mammalian target of rapamycin (mTOR) pathway in the skeletal muscle of normal-conditioned (NBCS) and over-conditioned (HBCS) cows (n = 18 per treatment). Symbols indicate a difference (*$P < 0.05$) between the groups at a given time. S6K1 = ribosomal protein S6 kinase, polypeptide 1; 4E-BP1 = eukaryotic translation initiation factor 4E binding protein. Data are presented as means ± SEM.

**Figure 5.** The mRNA abundance of genes related to the ubiquitin-proteasome system in the skeletal muscle of normal-conditioned (NBCS) and over-conditioned (HBCS) cows (n = 18 per treatment). Symbols indicate a difference (*$P < 0.05$) between the groups at a given time. UBA1 = ubiquitin-like modifier activating enzyme 1; UBE2G1 and UBE2G2 =

732 ubiquitin-conjugating enzymes; MuRF-1 = muscle ring-finger protein-1. Data are
733 presented as means ± SEM.

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**Table 1:** Ingredient composition and chemical composition (% of DM, unless otherwise noted) of rations during the observation period for cows of n
ormal-conditioned (NBCS) and high-conditioned (HBCS) cows

| | Late lactation | | Dry period | Early lactation |
|---|---|---|---|---|
| | 15 to 7 wk a.p.[1] | | wk 7 a.p. to parturition | 1 to 14 wk 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[2] | 0.4 | 0.7 | 0.7 | 0.4 |
| Concentrate[3] | 36.2 | 25.8 | 25.8 | 36.2 |
| | | | | |
| Analyzed chemical composition | | | | |
| ME (MJ/kg of DM) | 10.8 | 10.6 | 10.6 | 10.8 |
| $NE_L$ (MJ/kg of DM) | 7.2 | 6.8 | 6.8 | 7.2 |
| Crude protein (g/kg of DM) | 170 | 157 | 157 | 170 |
| Utilizable crude protein (g/kg of DM) | 156 | 149 | 149 | 156 |
| NDF[4] (g/kg of DM) | 359 | 382 | 382 | 359 |
| ADF[5] (g/kg of DM) | 204 | 223 | 223 | 204 |
| NFC[6] (g/kg of DM) | 402 | 360 | 360 | 402 |
| Ruminal N balance (g/d) | 3.4 | 2.3 | 2.3 | 3.4 |

[1]a.p. = Antepartum
[2]Contained (DM basis): 9.0% Ca, 9.0% P, 9.0% Na, 10% Mg, 10,000 mg/kg of Zn, 6,000 mg/kg of Mn, 1,500 mg/kg of Cu, 60 mg/kg of Co, 200 mg/kg of I, 53 mg/kg of Se, 1000 kIU/kg of vitamin A, 150 kIU/kg of vitamin $D_3$, 6 kIU/kg of vitamin E.
[3]Concentrate portion consisted of barley (25% of DM), corn grain (31% of DM), soybean meal (18% of DM), and canola meal (26% of DM)
[4]NDF = Neutral detergent fiber
[5]ADF = Acid detergent fiber
[6] NFC = Non-fiber carbohydrate

**Table 2.** Characteristics of primers and real-time PCR conditions.

| Gene[1] | Sequence (5'-3') | NCBI accession no. | bp | Annealing (s/°C)[2] | Mean efficiency |
|---|---|---|---|---|---|
| *mTOR* | | | | | |
| Forward | CGAAACCCTGGATGTCCCAA | XM_002694043.2 | 94 | 30/61 | 96.1 |
| Reverse | AGGACACCAGCCAATGTAGC | | | | |
| *4EBP1* | | | | | |
| Forward | CCCTGGAGGTACCAGGATCA | NM_001077893.2 | 125 | 60/62 | 103.3 |
| Reverse | CATCGCCTGTAGGGCTAGTG | | | | |
| *S6k1* | | | | | |
| Forward | CGGAACAGTCACACACACCT | NM_205816.1 | 97 | 30/61 | 100.1 |
| Reverse | ACTCCACCAATCCACAGCAC | | | | |
| *UBA1* | | | | | |
| Forward | GGGGAACCGGCATTGATGT | NM_001102477.1 | 118 | 60/59 | 92.2 |
| Reverse | AGGGCACTTCGGACAATACG | | | | |
| *UBE2G1* | | | | | |
| Forward | TATGCTGGCAGACCCCAATG | NM_001082458.1 | 109 | 60/59 | 103.3 |
| Reverse | TCTTACACAGCGGGCAACTT | | | | |
| *UBE2G2* | | | | | |
| Forward | TCACCCCAACATCTACCCAGA | NM_001076328.1 | 129 | 60/59 | 96.2 |
| Reverse | AGGAGGATCTTCTCCACGCT | | | | |
| *Atrogin-1* | | | | | |
| Forward | GAAACGCTTCCTGGACGAGA | NM_001046155.1 | 125 | 30/59 | 92.7 |
| Reverse | TCTTCTTGGCTGCAACGTCA | | | | |
| *MuRF-1* | | | | | |
| Forward | CCTGATCCAGGATGGAAACCC | NM_001046295.1 | 149 | 60/59 | 89.6 |
| Reverse | CAGCCTGCTGGAAGATGTCGT | | | | |
| *LRP10* | | | | | |
| Forward | CCAGAGGATGAGGACGATGT | BC149232 | 125 | 45/59 | 98.4 |
| Reverse | ATAGGGTTGCTGTCCCTGTG | | | | |
| *POL2RA* | | | | | |
| Forward | GAAGGGGGAGAGACAAACTG | X63564 | 86 | 60/60 | 100.4 |
| Reverse | GGGAGGAAGAAGAAAAAGGG | | | | |
| *EMD* | | | | | |
| Forward | GCCCTCAGCTTCACTCTCAGA | NM_203361 | 100 | 45/59 | 95.5 |
| Reverse | GAGGCGTTCCCGATCCTT | | | | |
| *EIF3K* | | | | | |
| Forward | CCAGGCCCACCAAGAAGAA | NM_001034489 | 180 | 60/59 | 89.8 |
| Reverse | TTATACCTTCCAGGAGGTCCATGT | | | | |

[1]*mTOR* = mammalian target of rapamycin; *4EBP1* = eukaryotic translation initiation factor 4E binding protein 1; *S6k1* = ribosomal protein S6 kinase, polypeptide 1; *UBA1* = ubiquitin-like modifier activating enzyme 1; *UBE2G1* = ubiquitin-conjugating enzyme E2G 1; *UBE2G2* = ubiquitin-conjugating enzyme E2G 2; *MuRF-1* = muscle ring-finger protein-1; *LRP10* = lipoprotein receptor-related protein 10; *POL2RA* = RNA polymerase II; *EMD* = Emerin; EIF3K = eukaryotic translation initiation factor 3 subunit K.

[2]Initial denaturation for 10 min at 90°C; denaturation for 30 s at 95°C; extension for 30 s at 72°C, except for *4EBP1*, *UBA1*, *UBE2G1*, *UBE2G2*, *MuRF-1* (60 s at 72°C), and *LRP10* (20 s at 72°C).

**Table 3.** Skeletal muscle AA concentrations (pmol/mg) of normal- (NBCS) and over-conditioned HBCS) cows during the observation period.

| Item (pmol/mg tissue) | Group | Days relative to calving | | | | SEM | *P*-value[1] | | |
|---|---|---|---|---|---|---|---|---|---|
| | | -49 | +3 | +21 | +84 | | G | T | G × T |
| Ala | HBCS | 715 | 777 | 723 | 768 | 26.4 | 0.46 | 0.26 | 0.56 |
| | NBCS | 715 | 780 | 613 | 704 | | | | |
| Arg | HBCS | 96.2 | 90.5 | 90.1 | 89.2 | 4.21 | 0.47 | 0.40 | 0.41 |
| | NBCS | 74.0 | 89.0 | 78.5 | 107.2 | | | | |
| Asn | HBCS | 79.9 | 85.5 | 91.0 | 101 | 4.29 | 0.20 | 0.38 | 0.91 |
| | NBCS | 62.5 | 83.9 | 80.1 | 84.4 | | | | |
| Asp | HBCS | 129 | 159 | 186 | 170 | 6.66 | 0.57 | 0.89 | 0.71 |
| | NBCS | 151 | 182 | 151 | 166 | | | | |
| Cys | HBCS | $31.3^{A*}$ | $26.1^{AB}$ | $24.7^{AB\dagger}$ | $32.3^{A}$ | 1.27 | 0.85 | 0.09 | 0.04 |
| | NBCS | $18.2^{b}$ | $25.8^{ab}$ | $34.1^{a}$ | $31.2^{ab}$ | | | | |
| Gln | HBCS | 3319 | 3257 | 3364 | 3369 | 138 | 0.28 | 0.90 | 0.98 |
| | NBCS | 2739 | 3081 | 3526 | 2945 | | | | |
| Glu | HBCS | 1969 | 1880 | 2180 | 2660 | 102 | 0.99 | 0.42 | 0.78 |
| | NBCS | 2168 | 2218 | 2230 | 2375 | | | | |
| Gly | HBCS | $1213^{\dagger}$ | 1086 | 979 | 1301 | 53.0 | 0.64 | 0.33 | 0.80 |
| | NBCS | 817 | 1028 | 1222 | 1057 | | | | |
| His | HBCS | $80.0^{B}$ | $99.1^{AB}$ | $105^{AB}$ | $108^{A}$ | 4.04 | 0.81 | 0.03 | 0.95 |
| | NBCS | $79.3^{b}$ | $99.2^{ab}$ | $110^{a}$ | $100^{ab}$ | | | | |
| Ile | HBCS | 117 | 108 | 112 | 104 | 4.29 | 0.41 | 0.90 | 0.85 |
| | NBCS | 107 | 110 | 116 | 111 | | | | |
| Leu | HBCS | 139 | 133 | 129 | 114 | 4.87 | 0.21 | 0.92 | 0.54 |
| | NBCS | 126 | 127 | 141 | 147 | | | | |
| Lys | HBCS | 115 | 111 | 116 | 111 | 7.44 | 0.86 | 0.28 | 0.75 |
| | NBCS | 111 | 118 | 101 | 158 | | | | |
| Met | HBCS | 32.6 | 33.1 | 32.0 | 27.3 | 1.41 | 0.19 | 0.64 | 0.47 |
| | NBCS | 32.9 | 34.8 | 32.5 | 39.1 | | | | |
| Orn | HBCS | 23.2 | 23.4 | 19.1 | 18.3 | 0.83 | 0.62 | 0.79 | 0.28 |
| | NBCS | 19.2 | 19.1 | 17.7 | 22.4 | | | | |
| Phe | HBCS | 60.7 | 60.9 | 54.7 | 50.8 | 2.14 | 0.30 | 0.80 | 0.66 |
| | NBCS | 55.2 | 58.6 | 60.5 | 64.0 | | | | |
| Pro | HBCS | 195 | 184 | 155 | 159 | 5.43 | 0.36 | 0.90 | 0.47 |
| | NBCS | 170 | 173 | 182 | 177 | | | | |
| Ser | HBCS | 216 | 211 | 195 | 238 | 8.60 | 0.33 | 0.55 | 0.87 |
| | NBCS | 185 | 198 | 208 | 182 | | | | |
| Thr | HBCS | 153 | 147 | 140 | 144 | 4.76 | 0.59 | 0.24 | 0.39 |
| | NBCS | 145 | 177 | 126 | 149 | | | | |
| Trp | HBCS | 23.6 | 23.6 | 22.6 | 23.5 | 0.63 | 0.55 | 0.85 | 0.86 |
| | NBCS | 22.8 | 24.0 | 23.9 | 25.7 | | | | |
| Tyr | HBCS | 61.1 | 61.5 | 66.3 | 58.9 | 1.99 | 0.26 | 0.69 | 0.56 |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| Val | NBCS | 64.5 | 68.4 | 61.9 | 68.5 | 6.20 | 0.22 | 0.93 | 0.76 |
| | HBCS | 215 | 209 | 212 | 199 | | | | |
| | NBCS | 214 | 208 | 227 | 232 | | | | |
| Total AA | HBCS | 8765 | 8880 | 9033 | 9901 | 262 | 0.42 | 0.45 | 0.79 |
| | NBCS | 7763 | 8871 | 9260 | 8980 | | | | |

Statistical comparisons: G = group effect; T = time effect; G × T = group × time interaction
Differences between the groups are indicated with asterisks (*) when $P \leq 0.05$ or a trend (†$P < 0.1$) at a given time point, respectively. Different capital letters (A, B) indicate differences between time points within HBCS cows; different lowercase letters (a, b) stand for differences between time points within NBCS cows.

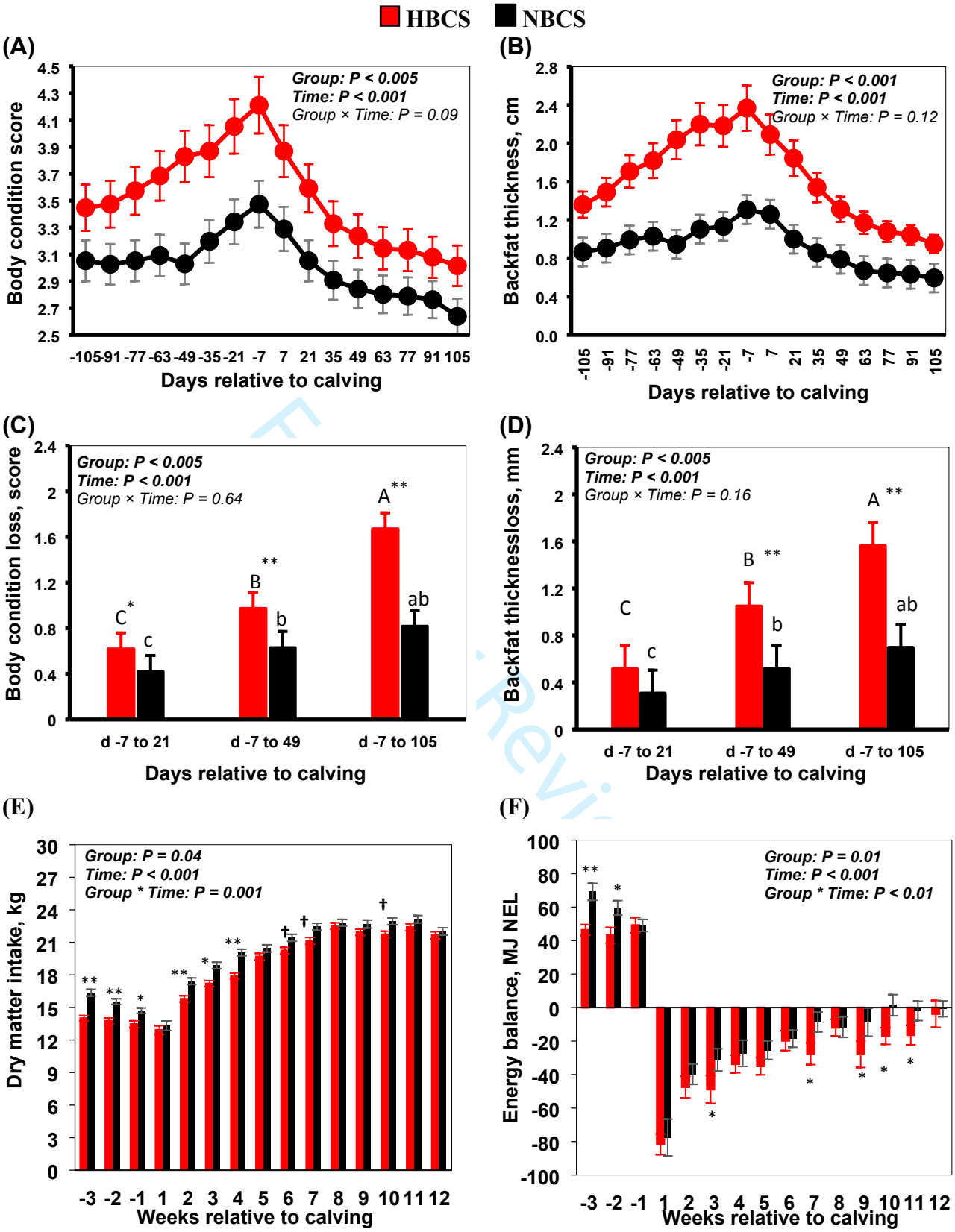
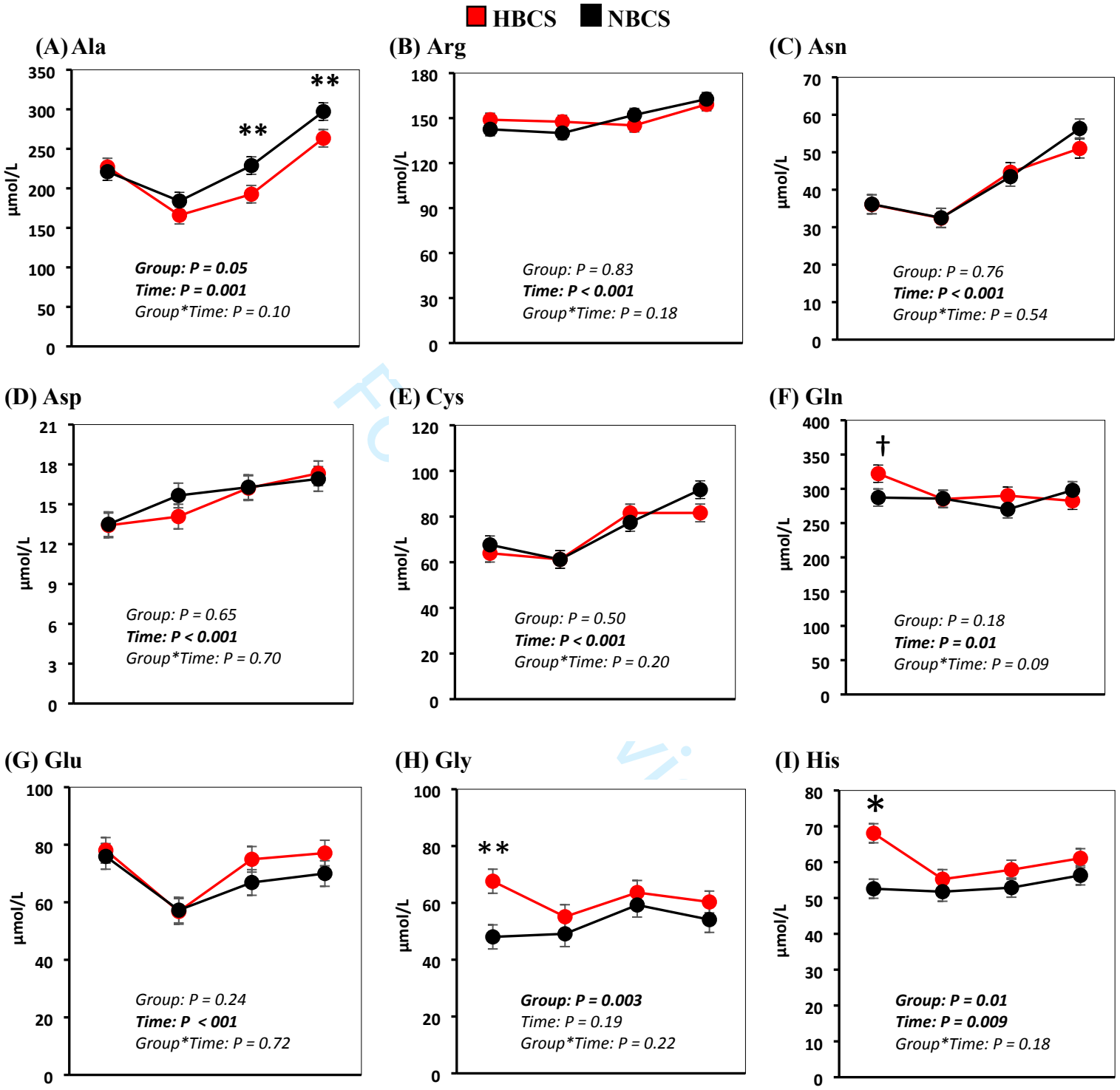



**Figure 1.**



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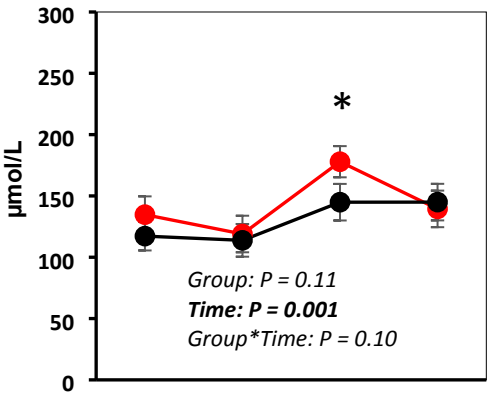


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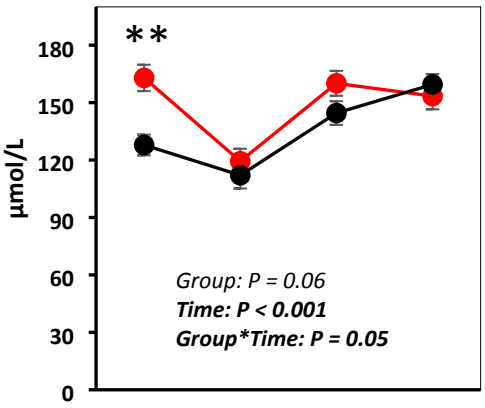


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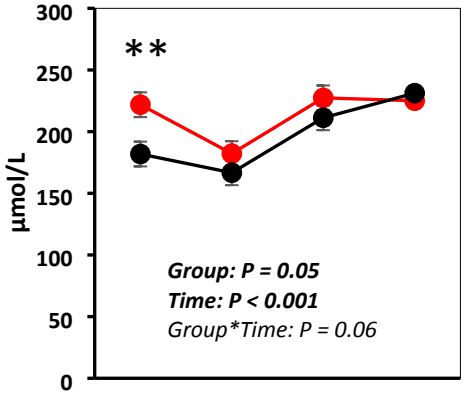
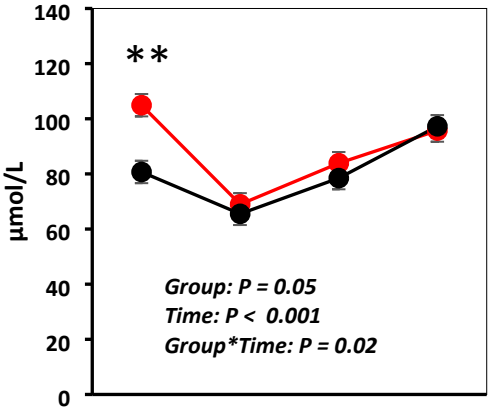



**(M) Lys**

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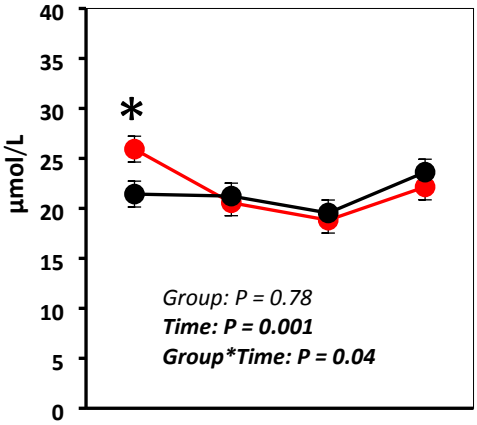


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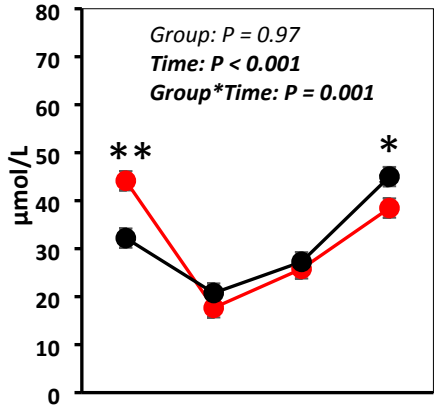
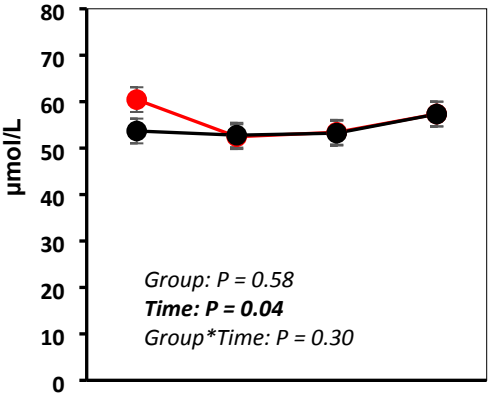



**(P) Phe**

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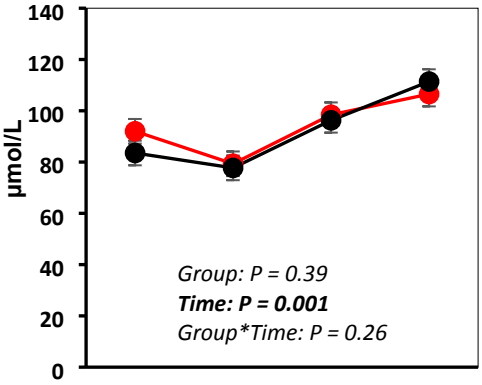
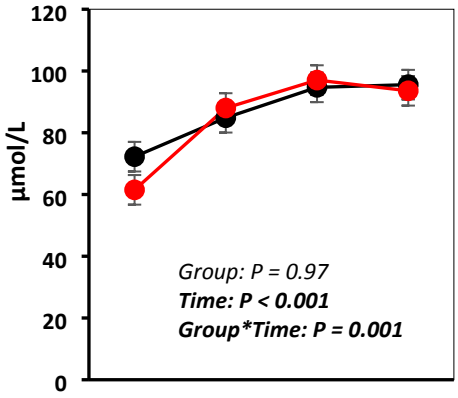
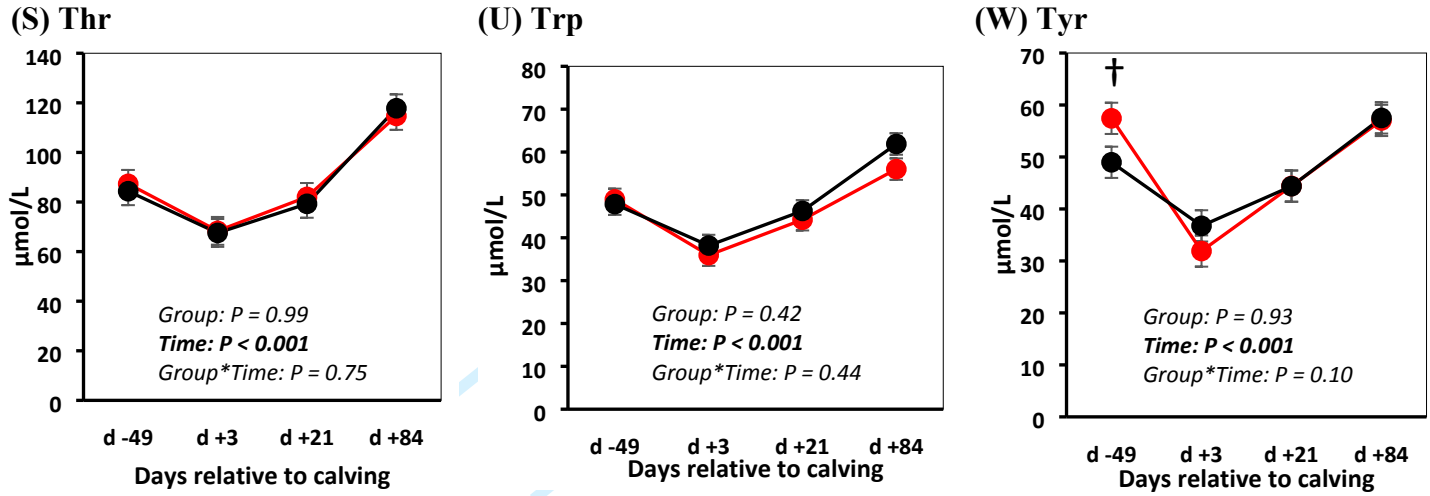



**(R) Ser**





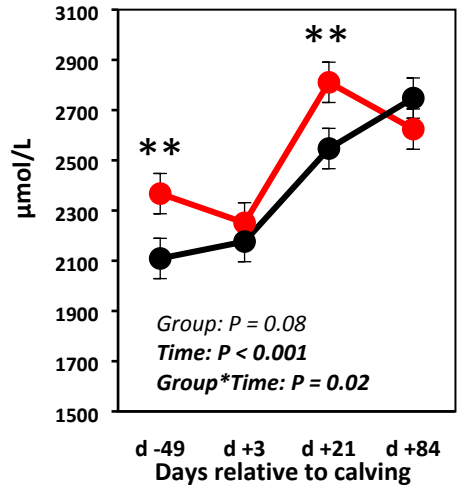




**Figure 2.**

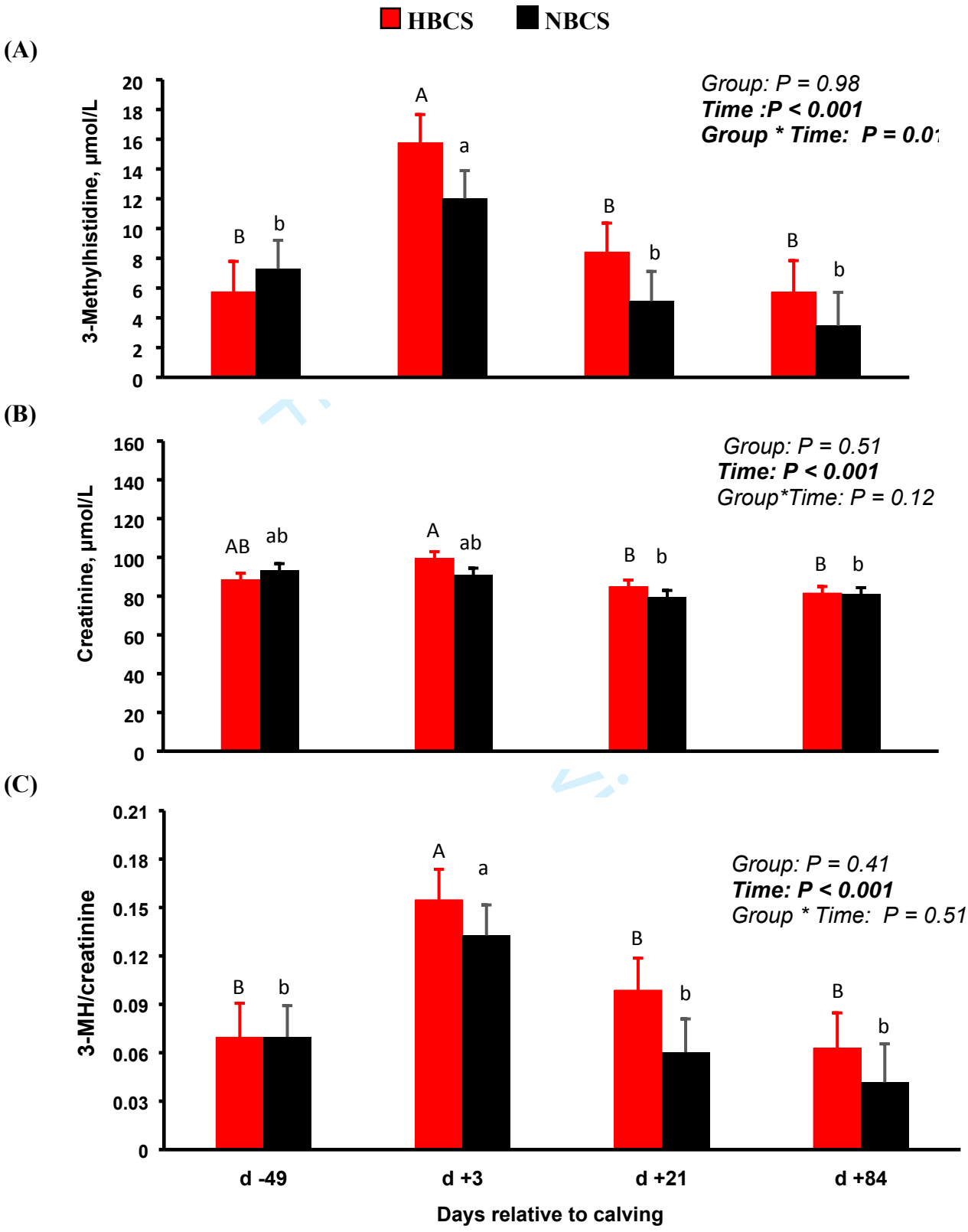



**Figure 3.**

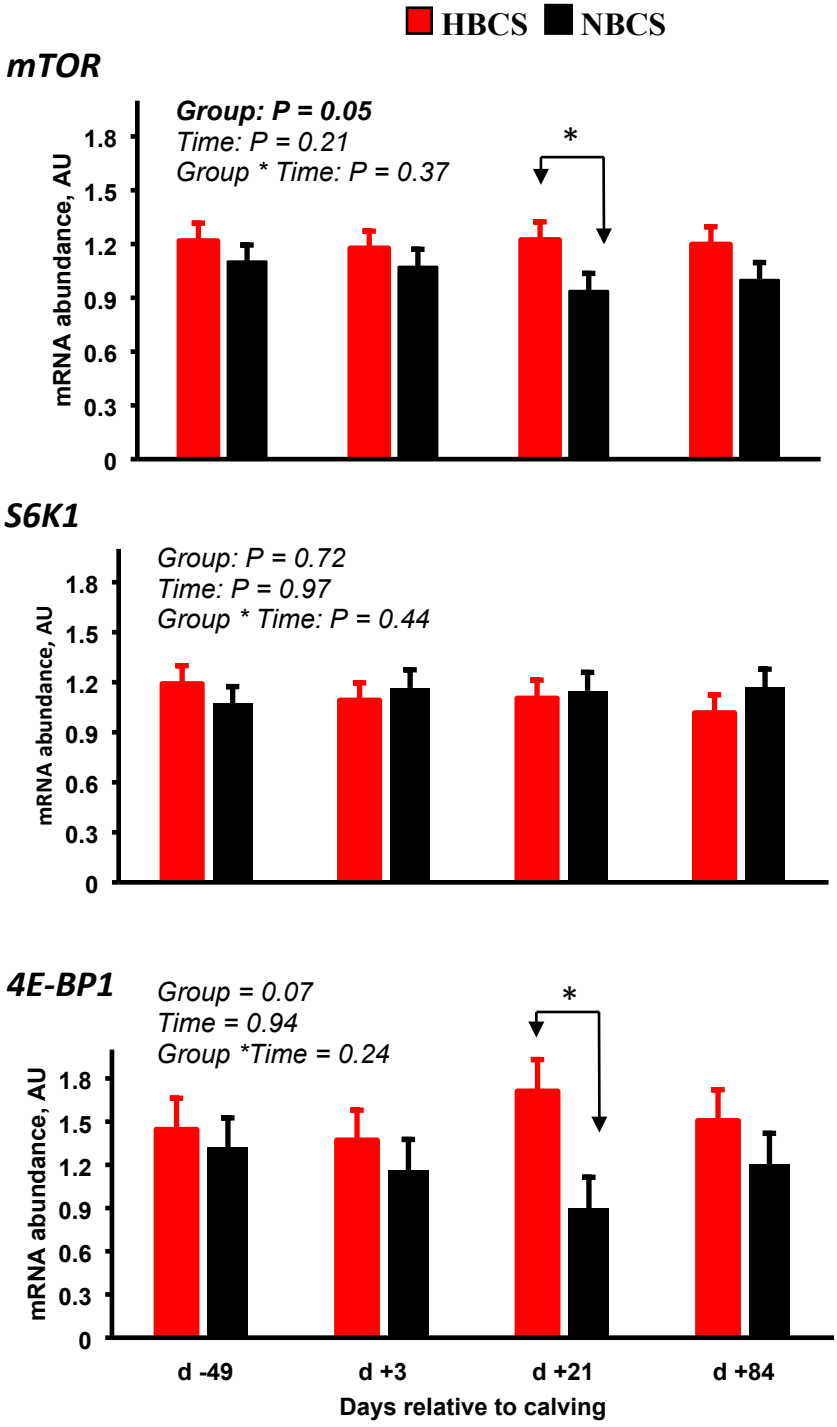



**Figure 4.**

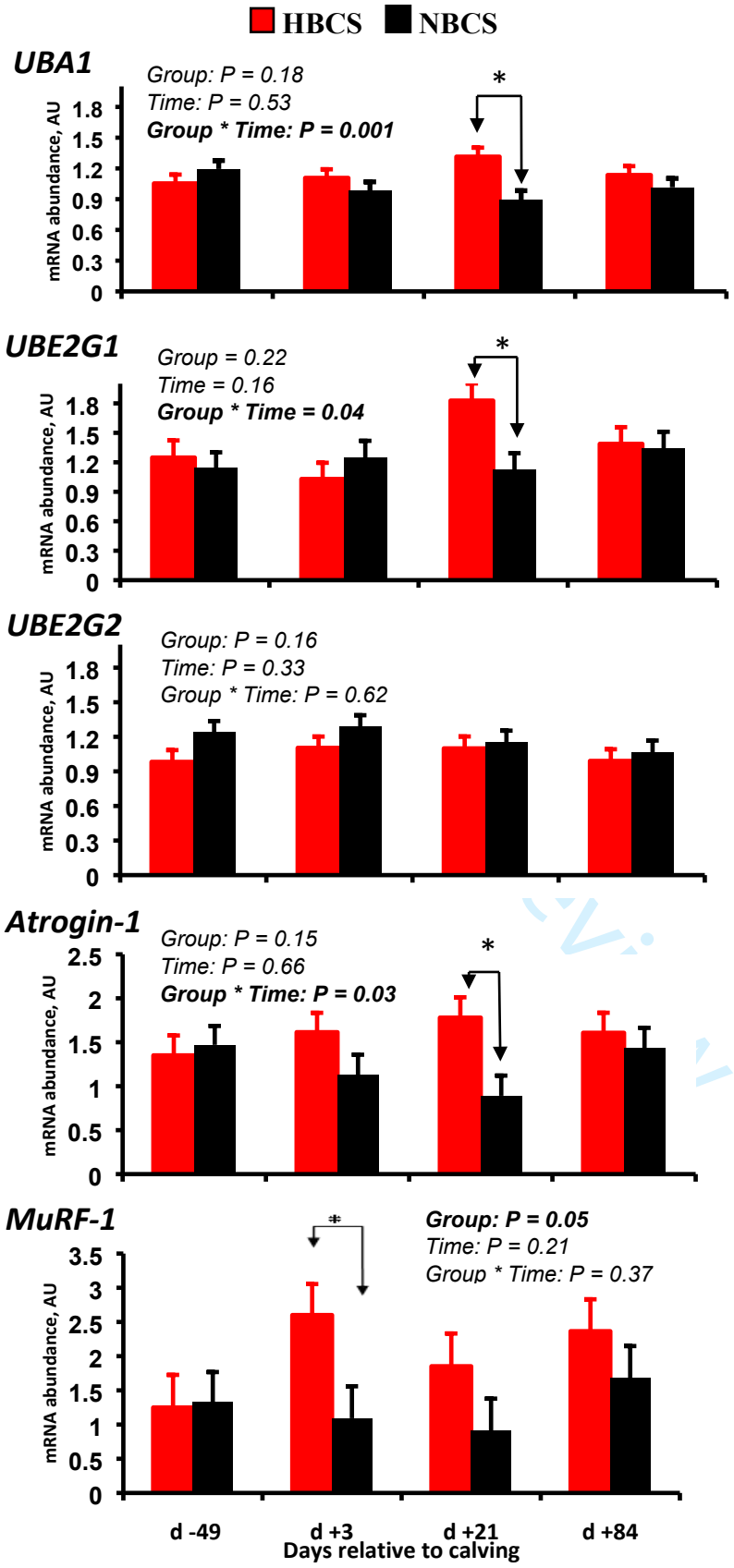
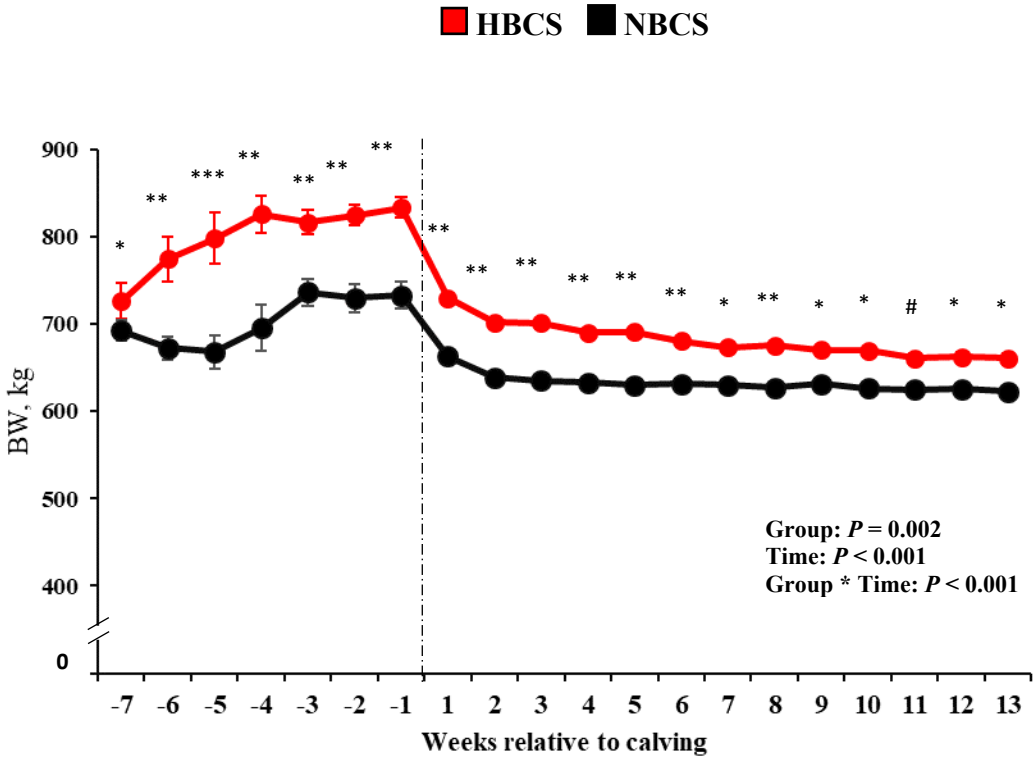



**Figure 5.**

## Supplemental Materials




**Supplemental Figure S1.** Changes of body weight (BW) in normal- (NBCS) and over-conditioned (HBCS) cows during the experimental period. Symbols indicate a difference (*$P < 0.05$; **$P < 0.01$) or a trend ($^{\#}P < 0.1$) between the groups at a given time-point. Data for BW are from Schuh et al. (2019). Data are presented as means ± SEM.

*Animal*, page 1 of 11 © The Animal Consortium 2019
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animal

# 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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*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* postpartum (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 $NE_L$ MJ/kg dry matter (DM); NBCS: 6.8 $NE_L$ 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.

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## Introduction

Overconditionend cows loose relatively more of their body condition in early lactation and have reduced dry matter 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 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 body condition score (NBCS) and one with high body condition score (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, $\beta$-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 with normal-weight human patients (Reinehr, 2010), we hypothesized that (d) HBCS cows might have elevated thyroid hormone concentrations around parturition.

## Material 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 *postpartum* (*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 $NE_L$ (MJ/kg of dry matter (DM)], whereas HBCS animals were fed the fresh cow ration with higher energy content [7.2 $NE_L$ (MJ/kg of DM)], from weeks 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 analysed according to the official recommendations of the Association of German Agricultural Analytic and Research Institutes (Naumann and Bassler, 2004). Samples were analysed for DM, crude ash, CP, utilizable CP, crude fat, crude fibre, ADF, NDF and non-fibre carbohydrytes, whereas the minerals calcium, phosphorus, magnesium, sodium and potassium were analysed by X-ray fluorescence analysis. The energy content of the diet (ME and $NE_L$) 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 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

**Table 1** *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 |
| Items | 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 (MJ/kg of DM) | 10.8 | 10.6 | 10.6 | 10.8 |
| $NE_L$ (MJ/kg of DM) | 7.2 | 6.8 | 6.8 | 7.2 |
| CP (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/day) | 3.4 | 2.3 | 2.3 | 3.4 |

*a.p.* = *Antepartum*; ME = metabolizable energy; $NE_L$ = net energy for lactation; 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), soya bean meal (18% of DM) and canola meal (26% of DM).

(Roughage Intake Control System; Insentec B.V., Marknesse, The 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 h in a milking parlour (GEA Farm Technologies GmbH, Boenen, Germany).

The calculations for the net energy requirement for maintenance ($NE_M$), pregnanacy and those for lactation ($NE_L$), as well as the milk energy concentrations were made according to the guidelines of the GfE (2001) as follows:

$NE_M$ (MJ $NE_L$/day) = 0.293 × $BW^{0.75}$;

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

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

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

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

Net energy balance (EB, MJ $NE_L$/day) = energy intake (MJ $NE_L$/day) − $NE_M$ (MJ $NE_L$/day) − $NE_L$ (MJ $NE_L$/day);

Energy intake = daily DMI × 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/day) = milk yield (kg/day) × [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 h; 50 : 50 vol/vol). Milk fat, protein, lactose, urea and somatic cell counts were assessed using a milk analyser based on Fourier transform IR spectroscopy (Bentley FTS; Bentley Instruments Inc., Chaska, MN, 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 CV were 6.3% and 13.9%, the standard curve reached from 0.11 to 27 ng/ml, and the measuring range was 0.3 to 7 ng/ml. The corresponding numbers for haptoglobin were 3.9% and 12.2%, the range of the standard curve was 0.012 to 9 μg/ml with a measuring range of 0.1 to 2 μg/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 to 7 ng/ml, respectively. Serum dROM were measured using *N,N*-diethyl-para-phenylendiamine as chromogene with the modifications of Regenhard *et al.* (2014); results are given as $H_2O_2$ equivalents; the intra- and inter-assay CV were 6.3% and 10.0%, respectively. Total FRAP was measured according to Benzie and Strain (1996), as the ability of serum to reduce $Fe^{3+}$ ($FeCl_3 \bullet 6\ H_2O$) to $Fe^{2+}$; values are given as μmol $Fe^{2+}$/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). 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 were >1.2 and >2.5 mM for clinical ketosis, 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 $P \leqslant 0.05$ and a trend was defined at $0.05 < P \leqslant 0.10$.

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 overconditioning 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 ($\Delta = 0.7$ 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

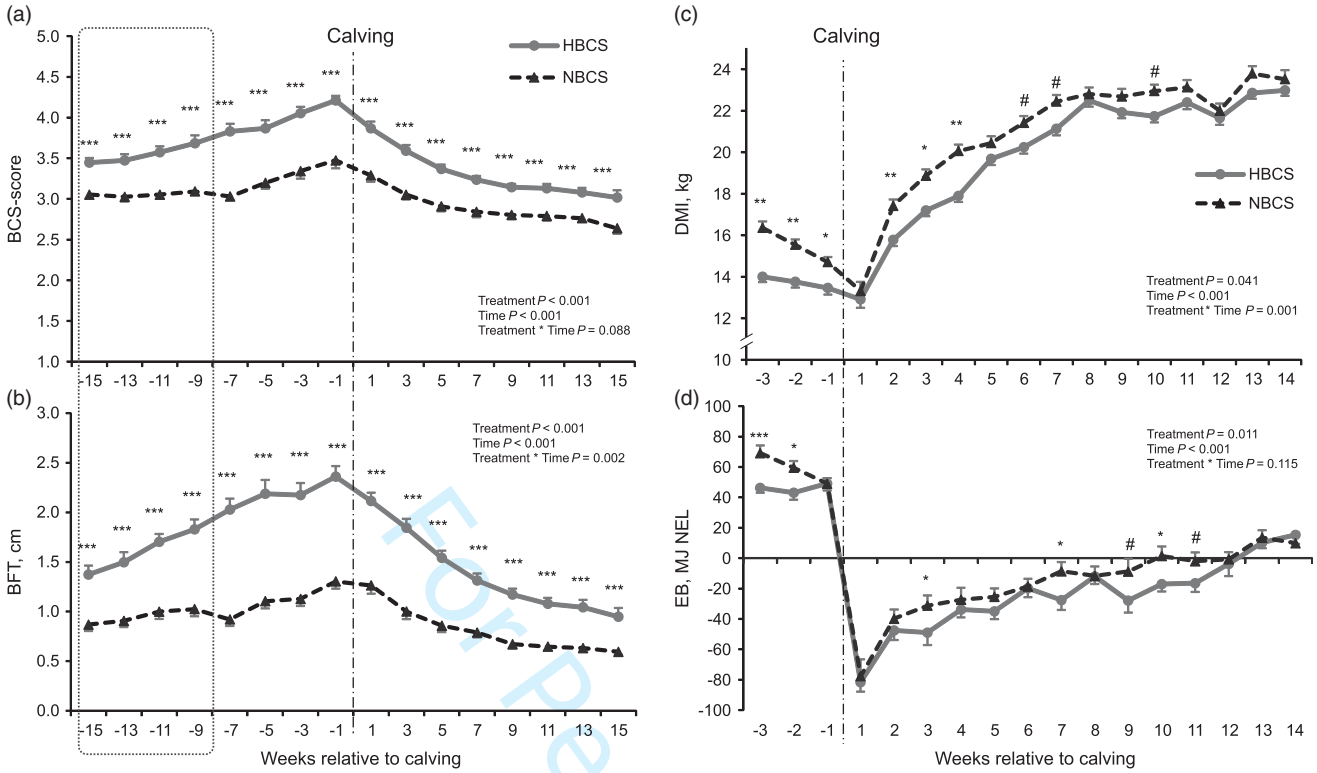


**Figure 1** Changes of (a) body condition score (BCS) and (b) back fat thickness (BFT) from 15 weeks *antepartum* (*a.p.*) to 15 weeks *postpartum* (*p.p.*) as well as (c) dry matter intake (DMI) and (d) energy balance (EB) from 3 weeks *a.p.* until 14 weeks *p.p.* (time = weeks relative to calving) 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 $P \leqslant 0.05$, or (**) when $P \leqslant 0.01$, or (***) when $P \leqslant 0.001$ at a given time point, respectively. Trends ($P \leqslant 0.10$) for differences between the groups at a given time point are indicated by (#).

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-day milk yield (weeks 1 to 14) was also the same in both groups (HBCS: 3816 ± 114 kg; NBCS: 3875 ± 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.

*Serum metabolites*

*Concentrations of non-esterified fatty acids, β-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

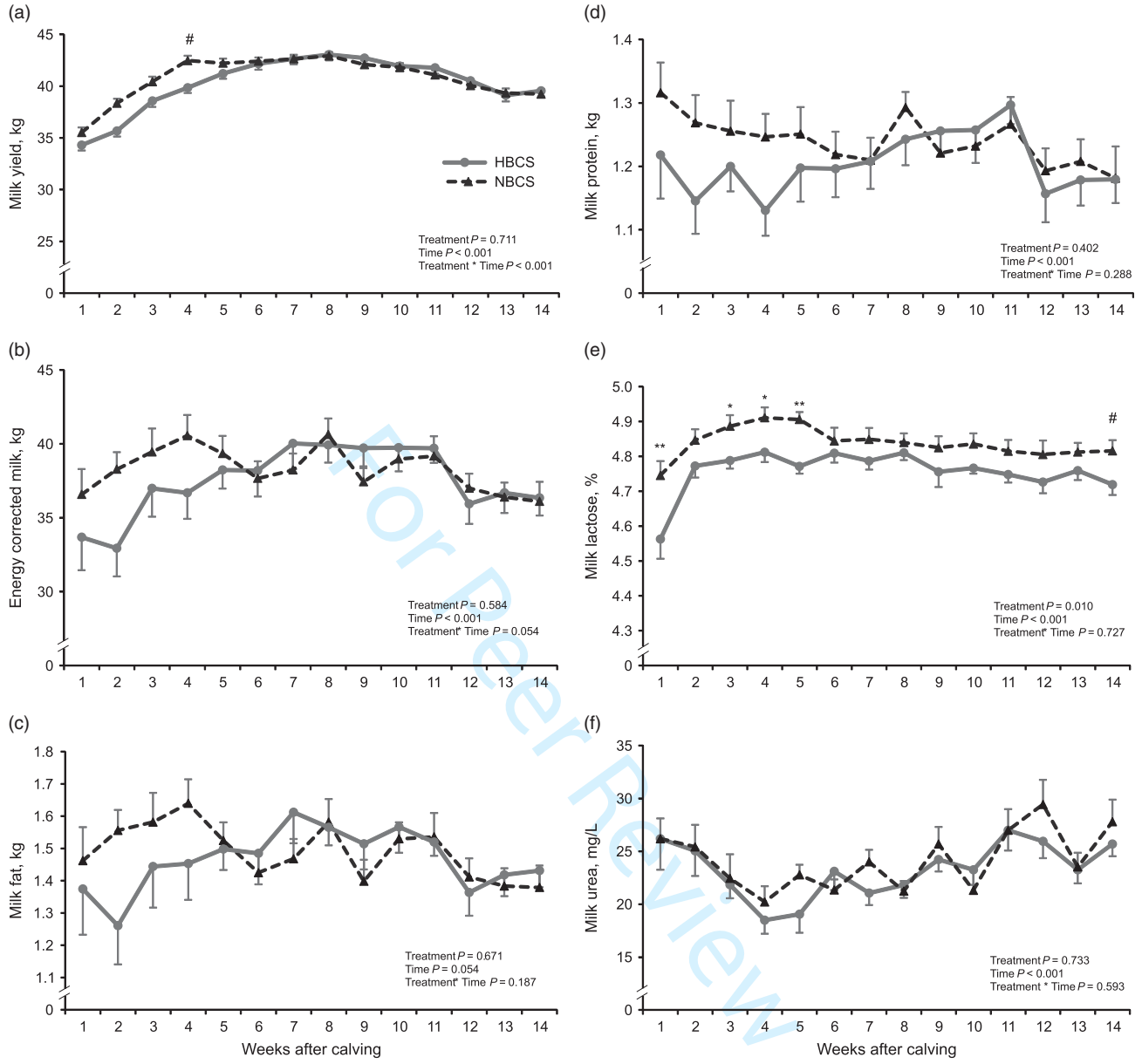


**Figure 2** Yields of (a) milk, (b) energy-corrected milk, (c) milk fat and (d) milk protein, and concentrations of (e) lactose and (f) urea in milk in high body condition score (HBCS) or normal body condition score (NBCS) cows from 1 to 14 weeks *postpartum* (time = weeks relative to calving). Results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

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 × 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% *v.* 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).

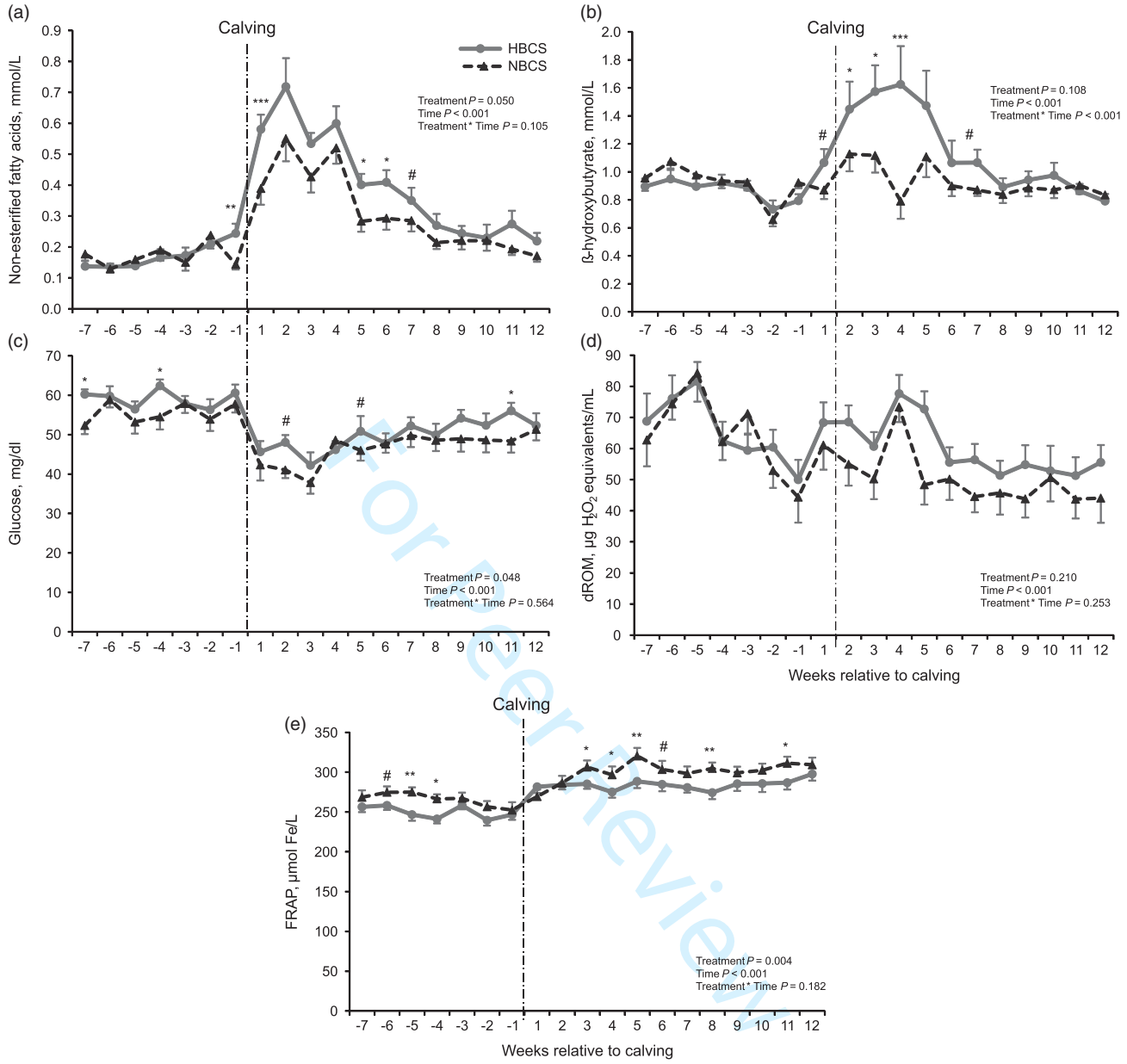


**Figure 3** Serum concentrations of (a) non-esterified fatty acids, (b) $\beta$-hydroxybutyrate, (c) glucose, (d) derivatives of reactive oxygen metabolites (dROM) and (e) ferric reducing ability of plasma (FRAP) in high body condition score (HBCS) or normal body condition score (NBCS) cows from 7 weeks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

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.

*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 IS 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 with thinner cows. As expected,

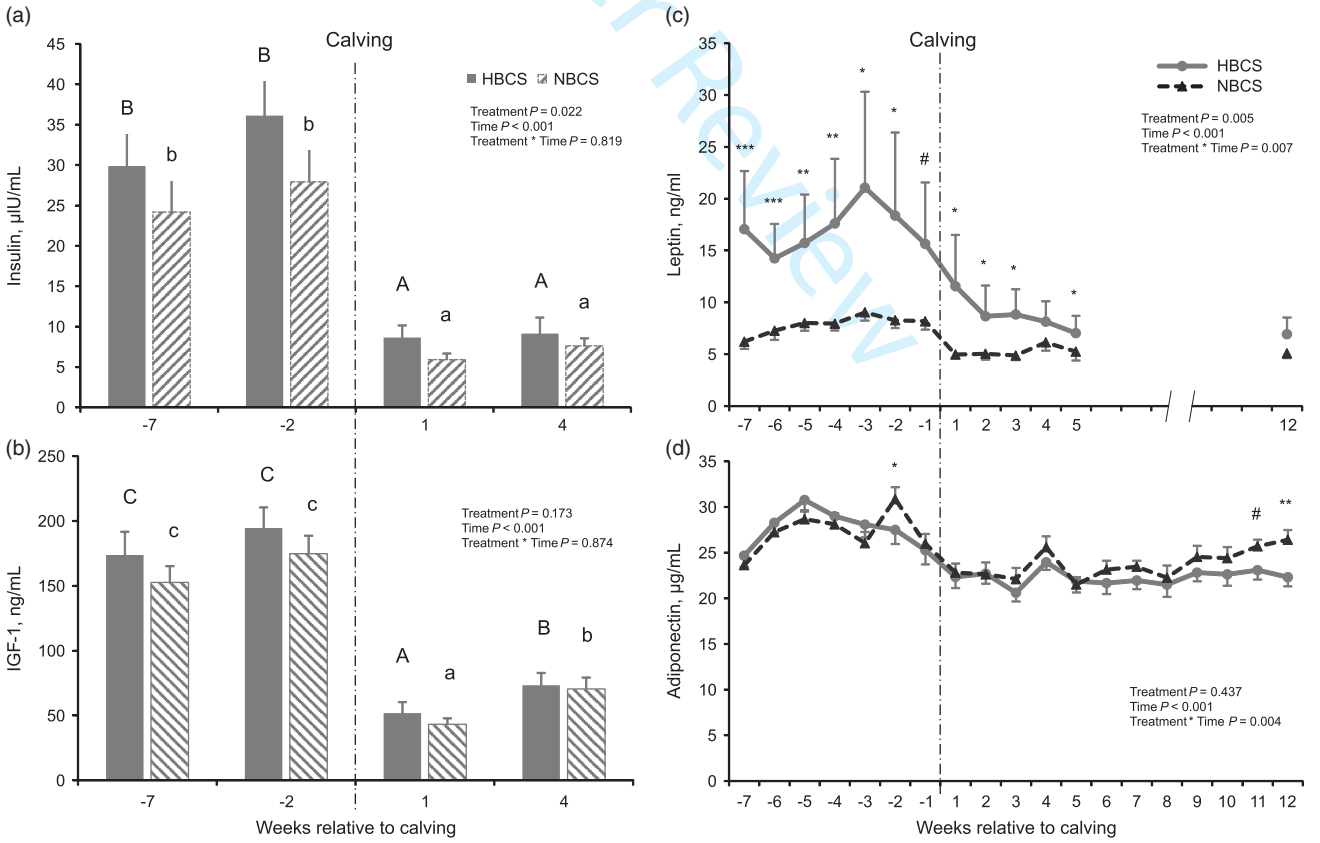


**Figure 4** Serum concentrations of (a) insulin, (b) IGF-1, (c) leptin and (d) adiponectin in high body condition score (HBCS) or normal body condition score (NBCS) cows (time = weeks relative to calving). Results are presented as means ± SEM. Significant differences between the groups are indicated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

circulating leptin was also correlated with BW and BCS ($r = 0.482$ and $r = 0.493$, respectively; $P < 0.001$).

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 BW 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

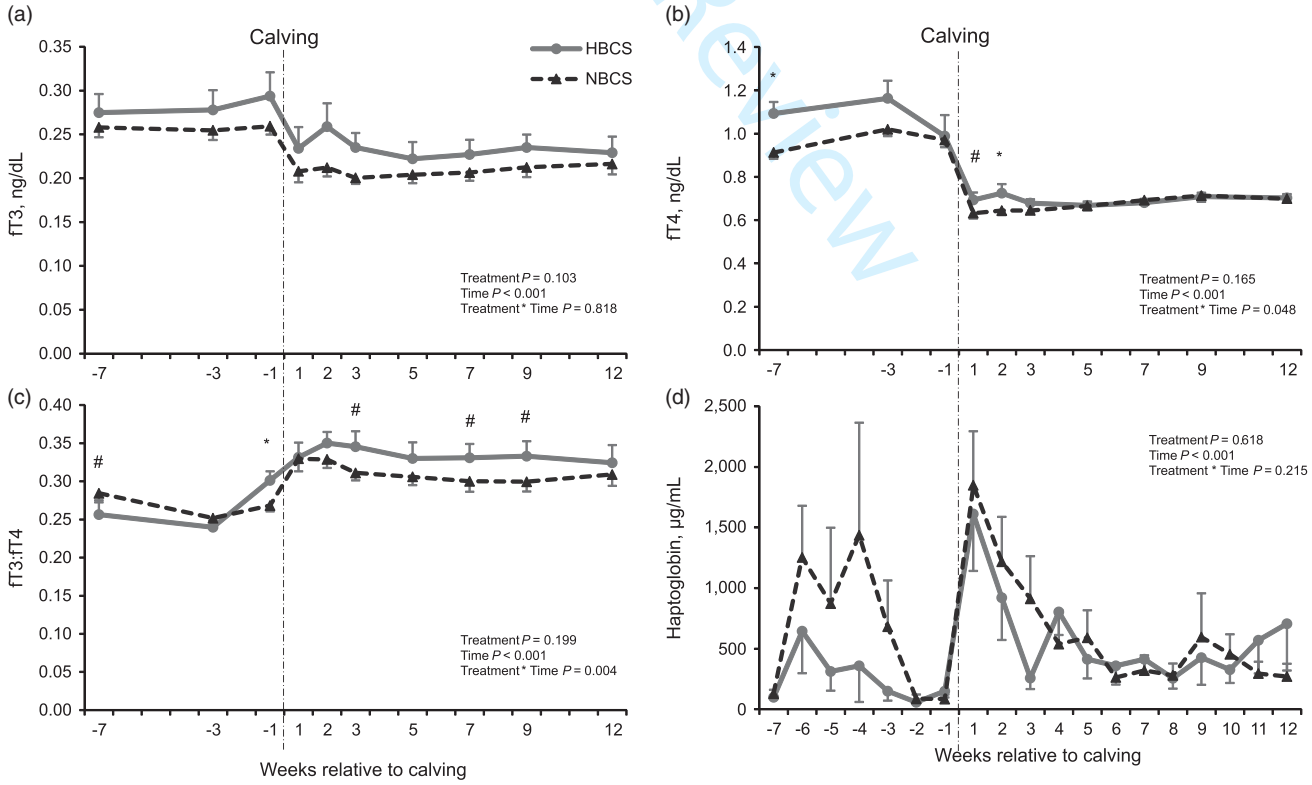


**Figure 5** Serum concentrations of (a) free triiodothyronine (fT3), (b) free thyroxine (fT4), (c) the ratio fT3/fT4 and of (d) haptoglobin in high body condition score (HBCS) or normal body condition score (NBCS) cows (time = weeks relative to calving). Results are presented as means ± SEM. Significant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

*et al*., 2007). Haptoglobin is mainly produced by the liver, but is also expressed in adipose tissue, undergoing similar changes as hepatic messenger RNA abundance and the circulating concentrations (Saremi *et al*., 2012). This time course was also observed in our study and without group differences (Figure 5d). 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). 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 ($r = 0.24$; $P \leqslant 0.05$). 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.

## 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 HBCS 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, HBCS 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 HBCS 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.

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## Declaration of interest

The authors declare that they have no conflicts of interest.

## Ethics statement

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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