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Molecular networks of insulin signaling and amino acid metabolism in subcutaneous adipose tissue are altered by body condition in periparturient Holstein cows

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ABSTRACT

Peripartal cows mobilize not only body fat but also body protein to satisfy their energy requirements. The objective of this study was to determine the effect of prepartum BCS on blood biomarkers related to energy and nitrogen metabolism, and mRNA and protein abundance associated with AA metabolism and insulin signaling in subcutaneous adipose tissue (SAT) in peripartal cows. Twenty-two multiparous Holstein cows were retrospectively classified into a high BCS (HBCS; n = 11, BCS ≥ 3.5) or normal BCS (NBCS; n = 11, BCS ≤ 3.17) group at d 28 before expected parturition. Cows were fed the same diet as a total mixed ration before parturition and were fed the same lactation diet postpartum. Blood samples collected at −10, 7, 15, and 30 d relative to parturition were used for analyses of biomarkers associated with energy and nitrogen metabolism. Biopsies of SAT harvested at −15, 7, and 30 d relative to parturition were used for mRNA (real time-PCR) and protein abundance (Western blotting) assays. Data were subjected to ANOVA using the MIXED procedure of SAS (v. 9.4; SAS Institute Inc., Cary, NC), with $P \leq 0.05$ being the threshold for significance. Cows in HBCS had greater overall plasma nonesterified fatty acid concentrations, due to marked increases at 7 and 15 d postpartum. This response was similar (BCS × Day effect) to protein abundance of phosphorylated (p) protein kinase B (p-AKT), the insulin-induced glucose transporter (SLC2A4), and the sodium-coupled neutral AA transporter (SLC38A1). Abundance of these proteins was lower at −15 d compared with NBCS cows, and either increased (SLC2A4, SLC38A1) or did not change (p-AKT) at 7 d postpartum in HBCS. Unlike protein abundance, however, overall mRNA abundances of the high-affinity cationic (*SLC7A1*), proton-coupled (*SLC36A1*), and sodium-coupled amino acid transporters (*SLC38A2*) were greater in HBCS than NBCS cows, due to upregulation in the postpartum phase. Those responses were similar to protein abundance of p-mTOR, which increased (BCS × Day effect) at 7 d in HBCS compared with NBCS cows. mRNA abundance of argininosuccinate lyase (*ASL*) and arginase 1 (*ARG1*) also was greater overall in HBCS cows. Together, these responses suggested impaired insulin signaling, coupled with greater postpartum AA transport rate and urea cycle activity in SAT of HBCS cows. An in vitro study using adipocyte and macrophage cocultures stimulated with various concentrations of fatty acids could provide some insights into the role of immune cells in modulating adipose tissue immunometabolic status, including insulin resistance and AA metabolism.

Key words: body condition, amino acid transporter, urea cycle, insulin resistance, subcutaneous adipose tissue

INTRODUCTION

Body condition, a subjective assessment of body energy reserves, has important implications for dairy cow management (Bewley and Schutz, 2008; Roche et al., 2013). Compared with cows calving at a normal BCS (**NBCS**), subcutaneous adipose tissue (**SAT**) of

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overconditioned cows (**HBCS**) has lower mRNA and protein abundance of the insulin receptor, along with more pronounced pro-inflammatory responses postpartum (Vailati-Riboni et al., 2016; Zhang et al., 2019). In contrast, mRNA abundance of AA transporters and phosphorylated (**p**) protein kinase B (AKT), a key regulator of the insulin signaling pathway, in SAT were greater in response to rumen-protected Met supplementation during the periparturient period (Liang et al., 2019). Thus, available data indicate that enhanced supply of certain AA during the periparturient period could help alleviate insulin resistance in SAT.

Human and rodent studies have revealed that insulin resistance and obesity are accompanied by increased circulating levels of branched-chain amino acids (**BCAA**; Newgard et al., 2009). Compared with muscle, liver, and mammary gland, adipose tissue (**AT**; visceral and subcutaneous) had the highest mRNA abundance of BCAA transaminase 2, suggesting that dairy cow AT might play a critical role in regulating BCAA catabolism in early lactation (Webb et al., 2019). Furthermore, compared with NBCS (BCS < 3.5) cows before calving, calving at HBCS (BCS > 3.75) led to greater abundance of BCAA catabolism-related molecules along with increased circulating concentrations of Leu, Ile, Val, His, Lys, and Orn (Ghaffari et al., 2019a). Altogether, these data suggest a potential relationship among body fatness—that is, degree of BCS—insulin signaling, and AA metabolism during the peripartal period.

The mechanistic target of rapamycin (mTOR), regulated by AA such as Leu, plays a crucial role in cellular growth, differentiation, and protein synthesis (Javed and Fairweather, 2019). Compared with NBCS cows, skeletal muscle of HBCS cows had greater mRNA abundance of *mTOR* and eukaryotic translation initiation factor 4E binding protein 1 (*EIF4EBP1*) without changes in ribosomal protein S6 kinase 1 (*RPS6KB1*), suggesting that BCS is associated with unique mTOR signaling pathway profiles (Ghaffari et al., 2019b). Our previous work indicated that enhanced postruminal supply of Met in peripartal cows resulted in greater concentrations of phosphorylated (activated) mTOR (p-mTOR), along with greater protein abundance of the Glu transporter SLC1A3 in SAT (Liang et al., 2019). Although solute carrier family 38 member 1 (SLC38A1), a neutral AA transporter, is responsible for Gln transport into cells (Mackenzie and Erickson, 2004), metabolism of Glu also can lead to synthesis of Gln via Gln synthetase (Palmieri et al., 2014). Beyond utilization for synthesis of cellular proteins, a recent study in rat white AT underscored the importance of a functional urea cycle (Arriarán et al., 2015) in the context of whole-body nitrogen metabolism. For instance, Gln is not only a critical oxidative fuel in cells (e.g., macrophages and neutrophils) but also a precursor for Orn, an important component of the urea cycle (Newsholme et al., 2003). Thus, changes in mRNA and protein abundance of mTOR components and AA transporters in AT might offers clues regarding novel aspects of nitrogen metabolism.

Our general hypothesis was that HBCS is associated with altered abundance of SAT and plasma biomarkers of energy and nitrogen metabolism during the periparturient period. Thus, the main objective of this study was to evaluate changes in mRNA and protein abundance of major components related to insulin signaling, AA transport, and urea cycle in SAT of peripartal cows with high or normal BCS in the late prepartum period.

MATERIALS AND METHODS

Experiment Design

All procedures involving animals were approved by the University of Illinois Institutional Animal Care and Use Committee (Urbana, IL; protocol #17168). Details of the experiment design were reported previously (Liang et al., 2020). Briefly, BCS was determined by 3 individuals weekly from −4 wk to 4 wk relative to expected calving date, and mean values of BCS were used for classifying cows in the present study. Twenty-two clinically healthy, multiparous Holstein cows were retrospectively classified into HBCS (3.75 ± 0.25, 3.5 to 4.0; mean ± SD; $n = 11$) or NBCS (3.07 ± 0.07, 3.0 to 3.17; mean ± SD; $n = 11$) groups on d 28 before expected parturition date, based on a 5-point scale (Edmonson et al., 1989). Dry cows were housed in a freestall barn with an individual Calan gate feeding system (American Calan, Northwood, NH). After calving, cows were housed in a tiestall barn. All cows had ad libitum access to a corn silage- and wheat straw-based TMR during the late prepartum period and a corn silage- and alfalfa hay-based TMR after parturition, with free access to water during the entire study. Diets were formulated to meet predicted requirements for dairy cows according to NRC (2001).

Blood Collection and Analyses

Blood obtained from the coccygeal vein before morning feeding, on d −10, 7, 15, and 30 relative to parturition, was collected into evacuated tubes containing lithium heparin (BD Vacutainer, Becton, Dickinson and Co., Franklin Lakes, NJ) and immediately placed on ice. Plasma was harvested by centrifugation at 2,000

× *g* for 15 min at 4°C, and aliquots were stored at −80°C until further analysis. Plasma concentrations of glucose, BHB, nonesterified fatty acid (**NEFA**), urea, and creatinine were determined following procedures described previously (Bionaz et al., 2007; Trevisi et al., 2012).

Adipose Tissue Biopsies

Cows in HBCS and NBCS averaged 28 ± 3 d in the close-up dry period. All (i.e., 11/group) were free of clinical disorders and had the full set of biopsies. Tissue was harvested from the tail-head (alternating between the right and left tail-head regions) at −15 (±2 d), 7, and 30 d relative to parturition, according to previous procedures from our laboratory (Ji et al., 2012). Briefly, the biopsy site was shaved, disinfected using iodine scrub and 70% alcohol, and anesthetized with 10 mL of 2% lidocaine HCl (VetOne, Boise, ID) before blunt dissection with sterile forceps and a scalpel. The incision was then closed with surgical staples (Henry Schein, Melville, NY), and iodine ointment (First Priority, Elgin, IL) was applied to the wound. Upon collection, AT was immediately placed in screw-capped microcentrifuge tubes, snap-frozen in liquid nitrogen, and preserved at −80°C until further analysis. Health was monitored for 7 d after surgery, and surgical clips were removed 7 d after the biopsy. No antibiotics were administered post-biopsy.

RNA Isolation, CDNA Synthesis, and Quantitative PCR

Total RNA isolation was as reported previously by our laboratory (Liang et al., 2020). Briefly, total RNA was isolated from 200 mg of SAT using the miRNeasy Kit (Qiagen, Hilden, Germany), according to the manufacturer's protocols. Samples of RNA were digested with DNaseI and quantified using a NanoDrop ND-1000 spectrophotometer (Thermo Fisher Scientific, Waltham, MA). Quality of RNA was measured using an Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA). Quantitative PCR was as described previously (Osorio et al., 2014). Validated internal controls were ribosomal protein S9 (*RPS9*), *GAPDH* and actin β (*ACTB*; Vailati-Riboni et al., 2015, 2016, 2017). Target genes and regulators associated with the insulin signaling pathway and nitrogen metabolism were based on previous studies (Takagi et al., 2008; Liang et al., 2019). Gene symbols, names, quantitative PCR performance, and primer information are included in Supplemental Table S1 (https://doi.org/10.3168/jds.2020-18612).

Western Blot Analysis

Approximately 100 mg of frozen SAT were homogenized with a MiniBeadBeater-16 (BioSpec Products, Bartlesville, OK) in 800 μL of Tissue Protein Extraction Reagent (catalog no. 78510; Thermo Fisher Scientific) containing Halt protease and phosphatase inhibitor cocktail (100×, catalog no. 78442; Thermo Fisher Scientific) for 60 s with 1 stainless steel bead (5 mm diameter; catalog no. 69989; Qiagen) and centrifuged at 10,000 × *g* for 15 min at 4°C. The fat layer was removed, and the supernatant was then carefully transferred into 1.5-mL tubes. Total proteins were extracted within 10 mo after sampling. The concentration of total protein was determined using the Pierce BCA protein assay kit (catalog no. 23227; Thermo Fisher Scientific). Details of the Western blot protocol were reported in our previous study (Liang et al., 2019). Briefly, protein samples were denatured by heating at 95°C for 5 min before loading 10 μL of protein into each lane of a 4–20% SDS-PAGE gel (catalog no. 4561096; Bio-Rad, Hercules, CA). Reactions were run for 10 min at 180 V, and then run for 45 to 60 min at 110 V. Then protein samples were transferred to a membrane in a Trans-Blot SD Semi-Dry Electrophoretic Transfer Cell (catalog no. 170-3940; Bio-Rad). Membranes were then blocked in 1× Tris-buffered saline (**TBST**) containing 5% nonfat milk for 2 h at room temperature. Membranes were then incubated in TBST containing primary antibodies to mTOR, p-mTOR (Ser2448), AKT, p-AKT (Ser473), solute carrier family 2 member 4 (SLC2A4, formerly GLUT4), solute carrier family 38 member 1 (SLC38A1), branched-chain α-keto acid dehydrogenase kinase (BCKDK; catalog no. and dilution ratio included in Supplemental Table S2, https://doi.org/10.3168/jds.2020-18612) overnight at 4°C. Antibodies for mTOR, p-mTOR (Ser2448), AKT, and p-AKT (Ser473) have been used previously in bovine tissues (Appuhamy et al., 2012; Zachut et al., 2013; Mann et al., 2016). SLC2A4, SLC38A1, and BCKDK are predicted to work with cows, and their predicted homology in bovine species is 100, 86, and 97%, respectively (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Furthermore, these antibodies were used successfully in our previous work with SAT (Liang et al., 2019). The dilution ratio of each antibody used in the current study was determined from 5 consecutive dilution ratios based on manufacturer's recommendations. Membranes were then washed with 1× TBST and incubated with anti-rabbit HRP-conjugated secondary antibodies (catalog no. 7074S, dilution 1:1,000; Cell Signaling Technology, Danvers, MA). Subsequently, membranes were washed with 1× TBST and then incubated with an enhanced

chemiluminescence reagent (catalog no. 170-5060; Bio-Rad) before image acquisition.

Visualized immunoblots were stripped and reprobed for target proteins and ACTB. ACTB is a well-established internal control for bovine AT (Locher et al., 2011; Zachut et al., 2013; Mann et al., 2016); hence, it was chosen (catalog no. 4967S; Cell Signaling Technology) for our previous (Liang et al., 2019, 2020) and current studies. Images were acquired using the ChemiDOC MP Imaging System (Bio-Rad). The intensities of the bands were measured with Image-Pro Plus 6.0 software (Media Cybernetics, Rockville, MD). Chemiluminescence signals were determined with at least 5 consecutive exposure times to ascertain the linear range of signal intensity of each antibody, to guarantee that quantitative data were obtained. Specific target protein band density values were normalized to ACTB density values. Representative blots are included in Supplemental Figure S1 (https://doi.org/10.3168/jds.2020-18612).

Statistical Analysis

The data were analyzed using the MIXED procedure of SAS version 9.4 (SAS Institute Inc., Cary, NC) according to the following model with repeated measures:

$$Y_{jl} = \mu + M_j + T_l + MT_{jl} + e_{jl},$$

where Y_{jl} = dependent, continuous variable, μ = overall mean, M_j = fixed effect of BCS (j = HBCS vs. NBCS), T_l = fixed effect of day (for blood parameter analysis, −10, 7, 15, and 30 d; for q-PCR and Western blot in SAT analysis, −15, 7, and 30 d), MT_{jl} = interaction between BCS and day, and e_{jl} = residual error. Cow, nested within BCS, was the random effect. The Kenward-Roger statement was used for computing the denominator degrees of freedom. The covariance structure of the repeated measurements was spatial power [SP(POW)]. When the interaction was significant, least squares means separation between and within time points was performed using the PDIFF statement with Tukey adjustment. Normality of the residuals was checked with normal probability and box plots, and homogeneity of variances was checked with plots of residuals versus predicted values. Significance was declared at $P \leq 0.05$ and tendencies at $P \leq 0.10$.

RESULTS AND DISCUSSION

Performance Responses

Production performance was reported previously (Liang et al., 2020). Briefly, both prepartum (12.0 vs. 12.3 kg/d) and postpartum (14.1 vs. 14.2 kg/d) DMI did not differ between HBCS and NBCS cows ($P > 0.05$); however, when expressed as percentage of BW, HBCS cows had lower prepartum DMI than NBCS (1.36 vs. 1.59; $P < 0.05$). Furthermore, no difference was detected in milk yield between HBCS (37.2 kg/d) and NBCS cows (38.2 kg/d; $P > 0.05$). Additionally, BCS or BCS × Day did not have an effect on milk composition and milk component yields (all $P > 0.05$; Table 1), which might partly be attributed to similar actual postpartum DMI between HBCS and NBCS cows.

Body Condition and Metabolic Status

Main effects of BCS, day, and their interaction on BW changes, and energy and nitrogen metabolism biomarkers in plasma are reported in Figures 1 and 2. Although we detected no effect of BCS or BCS × Day on plasma concentrations of glucose, BHB, cholesterol, and urea (all $P > 0.05$; Figure 2A, C, D, and E), which is similar to previous findings (Busato et al., 2002; Zhang et al., 2019). Those metabolites exhibited a sharp decrease or increase after parturition (Day, all $P < 0.01$; Figure 2A, C, D, and E), which was at least partly caused by decreased DMI and increased milk production. The HBCS cows had greater overall plasma NEFA concentration ($P < 0.01$; Figure 2B), which agrees with the results of Rico et al. (2015) and Jamali Emam Gheise et al. (2017). Because NEFA is one of

Table 1. Milk composition and milk component yields in Holstein cows with prepartum (28 d before expected parturition) high (HBCS, BCS ≥3.5) or normal BCS (NBCS, BCS ≤3.17) during the first 4 wk postpartum[1]

	BCS group			*P*-value		
Milk composition	HBCS	NBCS	SEM	BCS	Day	BCS × Day
Protein, %	2.95	3.08	0.08	0.23	<0.01	0.34
Protein, kg/d	1.19	1.22	0.13	0.90	0.39	0.61
Fat, %	3.25	3.35	0.40	0.87	0.36	0.10
Fat, kg/d	1.15	1.31	0.12	0.34	0.15	0.08
Lactose, %	4.78	4.85	0.05	0.24	0.02	0.11
Lactose, kg/d	1.96	1.99	0.17	0.90	<0.01	0.95

[1]Data are LSM, n = 11 cows per group, ± pooled SEM.

the major indicators of lipomobilization in dairy cows (González et al., 2011), greater plasma NEFA concentrations in HBCS cows underscored their noticeable fat mobilization during the periparturient period, which is in line with greater BW loss ($P < 0.01$; Figure 1). Despite the fact that we did not determine plasma insulin concentrations in the current study, previous research consistently reported that HBCS cows have plasma insulin levels similar to or greater than those of NBCS cows (Dann et al., 2005; Janovick et al., 2011; Pires et al., 2013; Alharthi et al., 2018; Schuh et al., 2019). Additionally, HBCS cows usually experience greater BW losses, along with decreased insulin sensitivity in SAT during the periparturient period (Zachut et al., 2013; Rico et al., 2015). Thus, plasma insulin alone is clearly not a "bullet-proof" biomarker for the assessment of insulin sensitivity in SAT.

Plasma creatinine is an index of muscle mobilization, and its excretion is proportional to body muscle mass (Ghaffari et al., 2019c; Megahed et al., 2019). Plasma creatinine concentration decreased after parturition irrespective of BCS (Day, $P < 0.01$; Figure 2F), which is consistent with the results reported by Ghaffari et al. (2019c) and Pires et al. (2013), indicating that dairy cows mobilize more body protein after parturition to compensate for decreased DMI and increased production energy requirements. Taken together, HBCS cows exhibited greater BW loss and mobilized more body fat to meet energy demands during the transition period. Although no effects due to body condition were detected, dairy cows appeared to mobilize muscle mass postpartum to meet energy requirements for milk production.

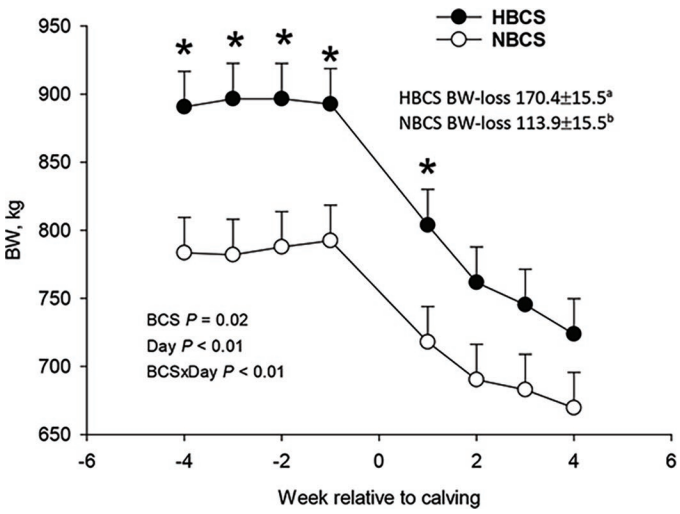

Figure 1. Change in BW and BW loss (kg) between −4 and 4 wk relative to parturition in Holstein cows with prepartum (28 d before expected parturition) high (HBCS, BCS ≥3.5) or normal BCS (NBCS, BCS ≤3.17). Data are LSM, n = 11 cows per group, ± pooled SEM. *Means differ (BCS × Day, $P \leq 0.05$) between groups (HBCS and NBCS). Groups with different lowercase letters differ ($P \leq 0.05$).

Insulin Signaling Components in Subcutaneous Adipose Tissue

Main effects of BCS, day, and their interaction for components of the insulin signaling pathway are reported in Figures 3 and 4. Compared with HBCS, NBCS cows had lower overall mRNA abundance of *AKT1* ($P = 0.04$; Figure 3A) and greater overall mRNA abundance of insulin receptor substrate 1 (*IRS1*; $P = 0.02$; Figure 3B). In NBCS cows, protein abundance of AKT decreased markedly from −15 to 7 d relative to calving, whereas during the same period, it increased slightly in HBCS cows (BCS × Day, $P < 0.01$; Figure 4A). A BCS × Day interaction was detected for protein abundance of SLC2A4, due to decreased protein abundance in NBCS cows and increased protein abundance in HBCS cows from −15 to 7 d relative to parturition ($P < 0.01$; Figure 4D). A murine study revealed that silencing peroxisome proliferator-activated receptor-γ (*PPARG*), a key regulator of lipid metabolism and glucose homeostasis, led to reduced SLC2A4 translocation in 3T3-L1 adipocytes (Liao et al., 2007), suggesting that *PPARG* activation contributes to the greater abundance of SLC2A4 in AT. We determined in a previous study that HBCS cows have greater mRNA abundance of *PPARG* in SAT (Vailati-Riboni et al., 2016), a response partly attributed to greater NEFA availability due to more pronounce lipolysis (Bionaz et al., 2013). Thus, we speculate that greater plasma concentrations of NEFA might promote *PPARG* activation in HBCS cows, subsequently influencing changes in SLC2A4 protein around parturition. Further research is warranted, to illustrate the role of *PPARG* in regulating glucose homeostasis in bovine SAT.

Insulin resistance, a key biological adaptation in peripartal cows, channels glucose to the placenta and the lactating mammary gland, but, if excessive, it can lead to greater incidence of metabolic disorders (Zhou et al., 2016). It is noteworthy that insulin resistance is more severe in SAT than in the liver during the periparturient period (Zachut et al., 2013), a response underscoring the essential role of SAT as an insulin-sensitive tissue in mammals. In AT, insulin binding to INSR triggers a cascade of events, including activation of IRS1 and phosphorylation of AKT (Saltiel and Kahn, 2001). A BCS × Day interaction was detected for *INSR* due to greater mRNA abundance in NBCS cows at d 7 postpartum ($P = 0.03$; Figure 3C), which is in line with the findings of Zhang et al. (2019). Such a response might have been partly responsible for greater activation of AKT (p-AKT/total AKT) at 7 d postpartum ($P <$

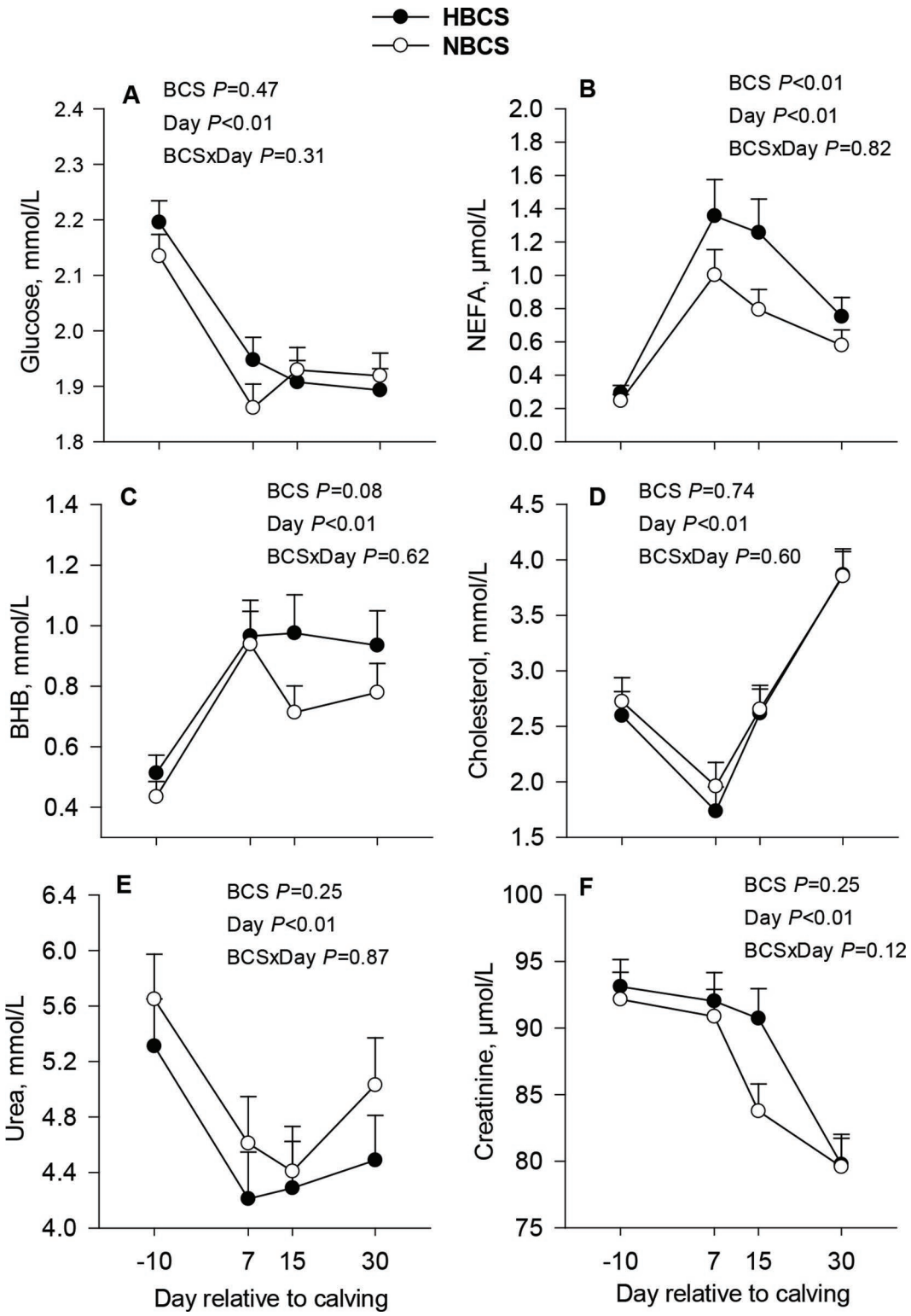

Figure 2. Plasma biomarkers of energy and nitrogen metabolism at −10, 7, 15, and 30 d relative to calving in Holstein cows with prepartum (28 d before expected parturition) high (HBCS, BCS ≥3.5) or normal BCS (NBCS, BCS ≤3.17). NEFA = nonesterified fatty acid. Data are LSM, n = 11 cows per group, ± pooled SEM.

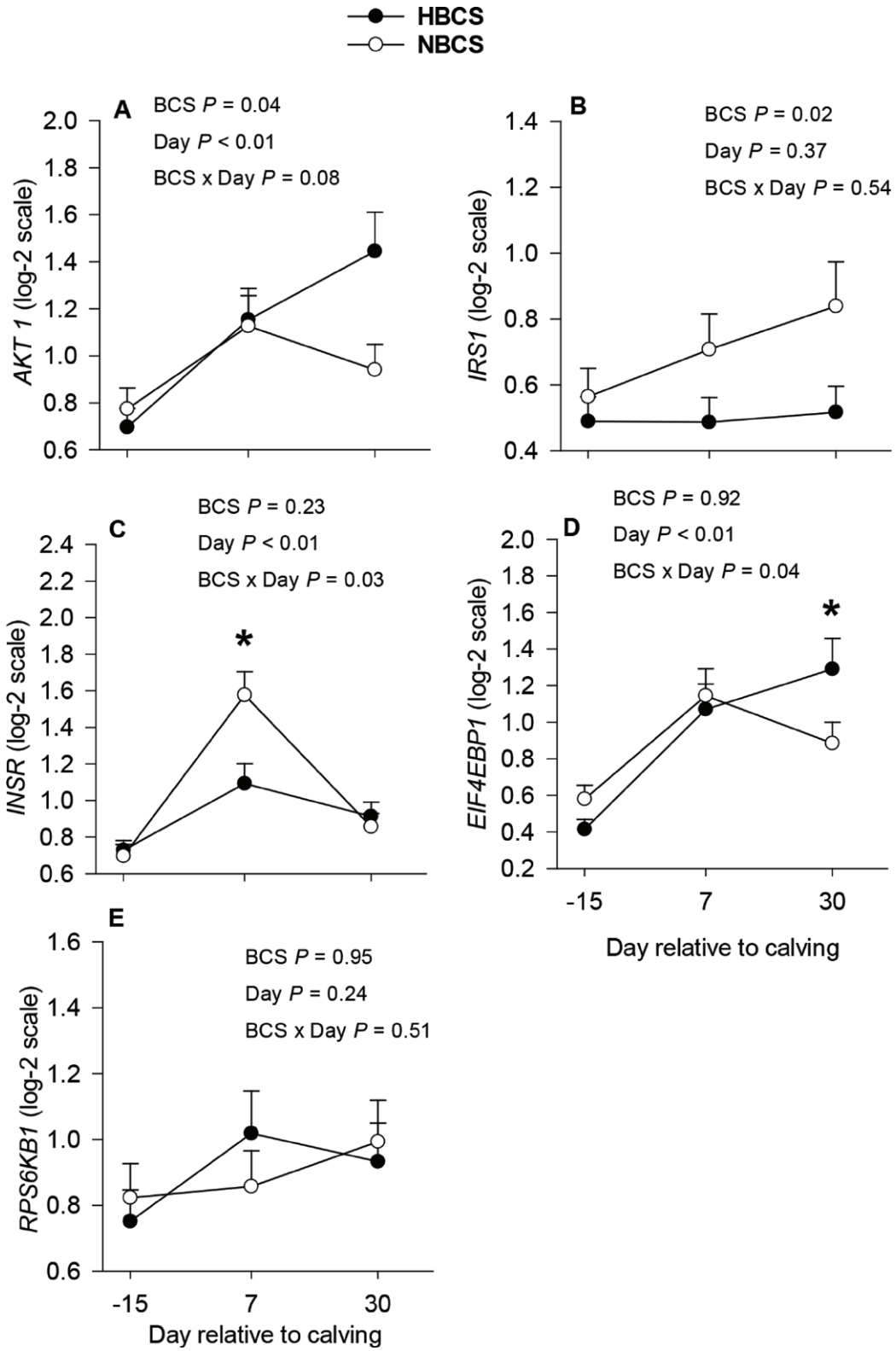

Figure 3. mRNA abundance of genes related to the mammalian target of rapamycin (mTOR) and insulin signaling pathways in subcutaneous adipose tissue of Holstein cows with prepartum (28 d before expected parturition) high (HBCS, BCS ≥3.5) and normal BCS (NBCS, BCS ≤3.17) at −15, 7, and 30 d relative to calving. Data are LSM, n = 11 cows per group, ± pooled SEM. *AKT1* = AKT serine/threonine kinase 1; *INSR* = insulin receptor; *IRS1* = insulin receptor substrate 1; *EIF4EBP1* = eukaryotic translation initiation factor 4E binding protein 1; *RPS6KB1* = ribosomal protein S6 kinase B1. *Means differ (BCS × Day, $P \leq 0.05$) between groups (HBCS and NBCS).

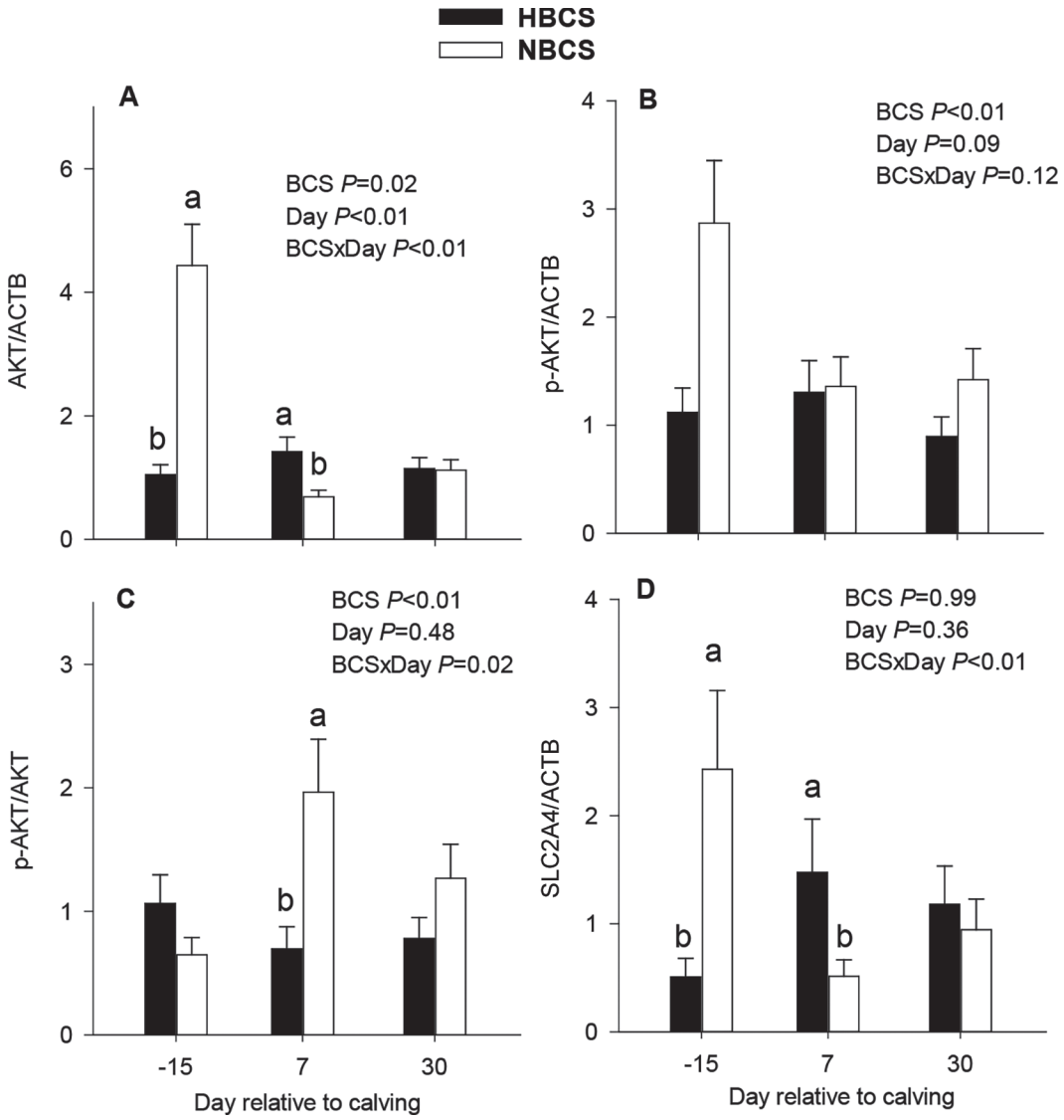

Figure 4. Protein abundance (relative to ACTB) of AKT (total, panel A), p-AKT (active, panel B), ratio of p-AKT/AKT (panel C), and SLC2A4 (panel D) in subcutaneous adipose tissue of Holstein cows with prepartum (28 d before expected parturition) high (HBCS, BCS ≥3.5) or normal BCS (NBCS, BCS ≤3.17) at −15, 7, and 30 d relative to calving. AKT = protein kinase B; SLC2A4 = glucose transporter 4. Data are LSM, n = 11 cows per group, ± pooled SEM. [a,b]Means differ (BCS × Day, $P \leq 0.05$).

0.01; Figure 4C). A downregulation of *IRS1* mRNA abundance was observed in murine adipocytes experiencing insulin resistance (Jager et al., 2007). Thus, the greater mRNA abundance of *IRS1* in NBCS compared with HBCS cows suggests NCBS cows were more sensitive to insulin.

Although a murine study demonstrated that AKT1 is mainly responsible for protein synthesis and cell survival, and AKT2 is involved in glucose homeostasis (Whiteman et al., 2002), the role of AKT isoforms in bovines is not well-documented (Ji et al., 2012). At least in vitro, both AKT1 and AKT2 can be activated by insulin (Gonzalez and McGraw, 2009), suggesting that these 2 isoforms are sensitive to insulin. Furthermore, both in vivo and in vitro studies have verified that cows with greater insulin sensitivity display greater activation of AKT (i.e., ratio of p-AKT to total AKT) in SAT (Zachut et al., 2013; Rico et al., 2018). Thus, greater ratio of p-AKT to total AKT in NBCS compared with HBCS cows ($P < 0.01$; Figure 4C) agrees with both greater mRNA abundance of *IRS1* and lower plasma NEFA, and suggests better insulin sensitivity in SAT. These data also agree with the fact that overconditioned cows exhibit decreased insulin sensitivity and reduced insulin responsiveness during late pregnancy (De Koster et al., 2015). Additionally, overconditioned

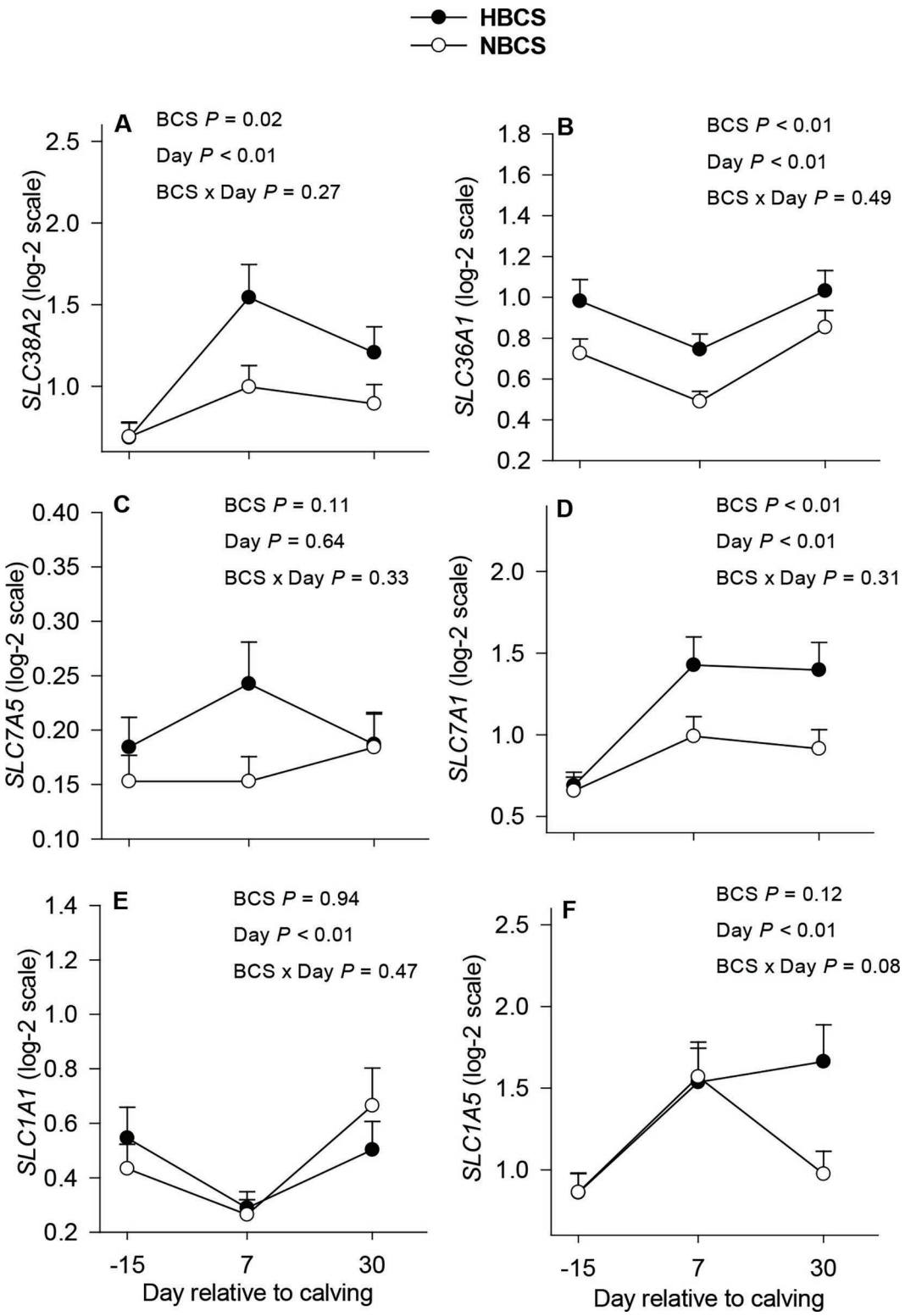

Figure 5. mRNA abundance of genes related to AA transport in subcutaneous adipose tissue of Holstein cows with prepartum (28 d before expected parturition) high (HBCS, BCS ≥3.5) and normal BCS (NBCS, BCS ≤3.17) at −15, 7, and 30 d relative to calving. Data are LSM, n = 11 cows per group, ± pooled SEM. *SLC1A1* = solute carrier family 1 member 1; *SLC1A5* = solute carrier family 1 member 5; *SLC7A5* = solute carrier family 7 member 5; *SLC7A1* = solute carrier family 7 member 1; *SLC36A1* = solute carrier family 36 member 1; *SLC38A2* = solute carrier family 38 member 2.

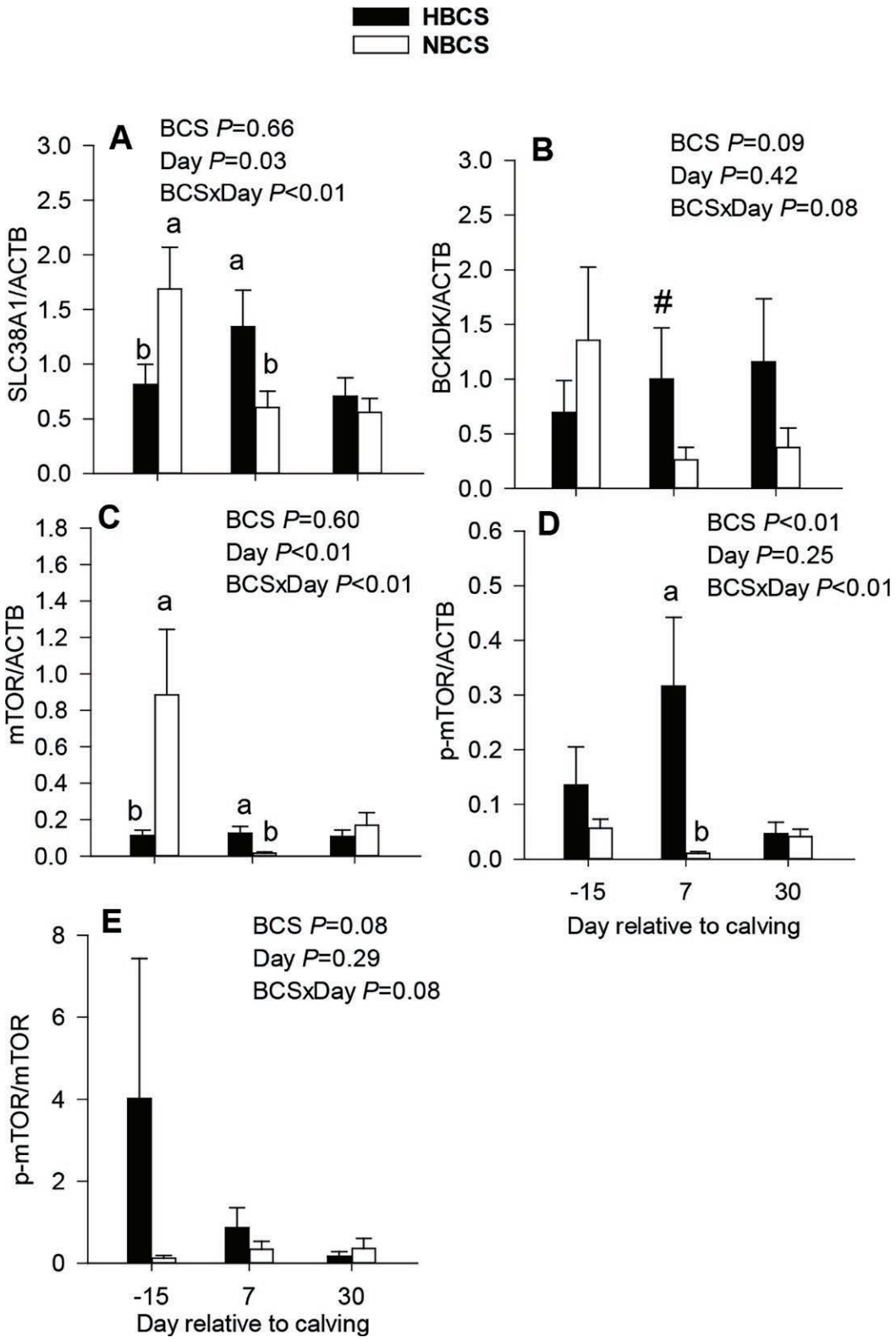

Figure 6. Protein abundance (relative to ACTB) of SLC38A1 (A), BCKDK (B), mTOR (total, C), p-mTOR (active, D) and ratio of p-mTOR/mTOR (E) in subcutaneous adipose tissue of Holstein cows with prepartum (28 d before expected parturition) high (HBCS, BCS ≥3.5) or normal BCS (NBCS, BCS ≤3.17) at −15, 7, and 30 d relative to calving. SLC38A1 = solute carrier family 38 member 1; BCKDK = branched-chain α-keto acid dehydrogenase kinase; mTOR = mechanistic target of rapamycin; p = phosphorylated. Data are LSM, n = 11 cows per group, ± pooled SEM. [a,b]Means differ (BCS × Day, $P \leq 0.05$). #Means tend to differ (BCS × Day, $P = 0.08$).

cows experience a greater degree of lipolysis between late pregnancy and early lactation (Rico et al., 2015), a response partly reflected by the greater BW loss they experience (Zachut et al., 2013). Thus, greater BW loss in HBCS cows after calving corresponds with other markers of insulin sensitivity and lipolysis.

In the context of AT function in the periparturient period, the fact that visceral AT and SAT have structural and functional differences (Ibrahim, 2010) underscores the need for additional mechanistic data in bovines. For instance, in obese humans and rodents, it is well established that macrophage infiltration in SAT leads to impaired insulin sensitivity (Kanda et al., 2006). Molecular data at the mRNA and protein level have revealed some differences in metabolism and immune responsiveness among bovine SAT and visceral AT depots (Ji et al., 2014a; Contreras et al., 2015; Kenéz et al., 2019). Although reports conflict (Akter et al., 2012; Häussler et al., 2017), recent data indicate that macrophage infiltration occurs in dairy cow SAT during the transition period and is positively associated with body fat mobilization (De Koster et al., 2018; Newman et al., 2019). Thus, HBCS cows might be more prone to macrophage infiltration in SAT, contributing to alterations in insulin sensitivity. These data underscore the need for further research; for instance, an in vitro study using adipocyte and macrophage cocultures challenged with increasing levels of NEFA could expand our understanding the role of macrophages in modulating tissue insulin sensitivity.

AA Transporters, Inflammation, and Oxidative Stress

Main effects of BCS, day, and their interaction, for AA transporters and mTOR, are reported in Figures 5 and 6. Compared with NBCS, HBCS cows had greater overall mRNA abundance of sodium-coupled AA transporter (*SLC38A2*), proton-coupled AA transporter (*SLC36A1*), and high-affinity cationic transporter (*SLC7A1*), mainly due to their upregulation postpartum ($P = 0.02$; $P < 0.01$; $P < 0.01$; Figure 5 A, B, and D). A BCS × Day interaction occurred for protein abundance of SLC38A1 due to decreased protein abundance in NBCS cows and increased protein abundance in HBCS cows from −15 to 7 d relative to parturition ($P < 0.01$; Figure 6A). This effect was similar to the pattern of SLC2A4. Furthermore, a BCS × Day interaction was observed for protein abundance of p-mTOR due to greater values at 7 d postpartum ($P < 0.01$; Figure 6D) in HBCS cows.

Amino acid transporters control uptake and flow across plasma membranes (Closs et al., 2006; Sundberg et al., 2008). For instance, SLC38A2 belonging to AA transport system A transports Asn, His, Ser, Cys, Met, and Gln (Mackenzie and Erickson, 2004; Menchini and Chaudhry, 2019). SLC7A1 plays a key role in Arg metabolism (Wang et al., 2014). SLC36A1 is an efficient carrier of Gly, Pro, and Ala (Bröer, 2008). An in vivo study in humans demonstrated that SAT contributes to net Ala and Gln production and Glu uptake during fasting (Frayn et al., 1991). Thus, available data suggest that AA transporters in SAT play an important role during normal tissue metabolism or in response to changes in nutrient supply.

Despite the fact that research related to AA metabolism in bovine SAT is still in its infancy, our recent studies established an association between enhanced postruminal supply of Met, greater DMI (Batistel et al., 2017) and plasma Met (Vailati-Riboni et al., 2019), and upregulated mRNA abundance of the AA transporters *SLC38A1*, Glu (*SLC1A1*), and *SLC1A5* in SAT (Liang et al., 2019). Thus, enhanced postruminal supply of AA might increase SAT utilization of AA partly through adaptations at the molecular level. Our previous work also confirmed that neutral AA (*SLC1A5*), BCAA (*SLC7A5*), and *SLC38A1* transporters are expressed in bovine SAT (Liang et al., 2019). In addition, compared with liver and skeletal muscle, bovine SAT has the greatest mRNA abundance of the BCAA transporters *SLC1A5* and *SLC7A5*, and branched-chain aminotransferase 2 (Webb et al., 2020). Thus, available data suggest that AA uptake and metabolism in SAT are important aspects of tissue metabolism.

Overall mRNA abundances of *SLC7A5*, *SLC1A1*, and *SLC1A5* were not affected by BCS ($P = 0.11$; $P = 0.94$; $P = 0.12$; Figure 5C, E, and F), similar to the results reported by Webb et al. (2020), indicating that BCAA uptake in SAT might not be affected by BCS. This is partly supported by the lack of differences in overall plasma BCAA and Glu concentrations (Ghaffari et al., 2019b; Webb et al., 2020). Furthermore, given the fact that SAT might be a major site for BCAA uptake and catabolism (Webb et al., 2019, 2020), the similar overall protein abundance of BCKDK in SAT between HBCS and NBCS cows ($P = 0.09$; Figure 6B) coincided with the finding that overall plasma BCAA concentrations are independent of BCS (Webb et al., 2020). Of particular interest, HBCS cows had greater serum and plasma concentrations of Ile or Leu than NBCS cows did on d +21 relative to calving (Ghaffari et al., 2019a,b; Webb et al., 2020).

The fact that HBCS cows had greater protein abundance of BCKDK at d 7 postpartum, along with plasma data from Ghaffari et al. (2019a,b), provides further support for the notion that SAT might play a critical role in regulating BCAA metabolism. However,

we draw this conclusion cautiously, due to the fact that ruminal microbiota are important contributors of circulating BCAA (Xue et al., 2020). In this context, it is noteworthy that human and rodent studies revealed that gut microbiota associated with BCAA de novo synthesis are enriched in obese individuals and are associated with development of insulin resistance in mice (Newgard, 2017). Thus, it is plausible that altered circulating BCAA levels in cows with different BCS might be modulated by the gut microbiome. The association between gut microbiome and plasma BCAA concentrations in peripartal dairy cows merits further study, as it might help elucidate the role of the gut in regulating insulin resistance.

Nonruminant studies indicate that restriction or underfeeding of dietary protein or AA can also control mRNA abundance of AA transporters in different tissues. For instance, reduced dietary protein supply increased mRNA abundance of *SLC7A1* and *SLC38A2* in the longissimus dorsi of pigs (Wang et al., 2017). Similarly, a low-protein diet led to increased mRNA abundance in pig SAT of the AA transporters *SLC7A5* and *SLC38A2* (Li et al., 2016). Challenges to the immune system also alter tissue AA utilization, such as the acute-phase response channel AA, toward synthesis of acute-phase proteins (Le Floc'h et al., 2004). Although the liver is the key organ where acute-phase proteins are synthesized, evidence suggests that cow AT (Saremi et al., 2012) also produces some acute-phase proteins. Thus, undersupply of AA or immune challenges associated with parturition can signal upregulation of certain AA transporters. The only difference in performance we detected was lower prepartal DMI as percent of BW in HBCS cows; thus, DMI per se cannot explain changes in AA transporters, which occurred primarily in the postpartum. It is possible that the greater inflammatory status we detected in HBCS cows (Liang et al., 2020) had an indirect effect on upregulation of AA transporter mRNA abundance in HBCS cows. Further in vitro research using an inflammatory challenge such as hydrogen peroxide (Ma et al., 2019) or lipopolysaccharide (Mukesh et al., 2010) could provide additional information in this regard.

Human studies have demonstrated that hypoxia contributes to insulin resistance and inflammation, partly due to dysregulated adipokine production in SAT of obese individuals (Hosogai et al., 2007; Trayhurn, 2013). Adiponectin (ADIPOQ), an important adipokine with anti-inflammatory and insulin-sensitizing functions, is primarily derived from white AT, and decreased ADIPOQ concentration in humans leads to tissue inflammation via enhancement of lipolysis (Ruan and Dong, 2016). Serum ADIPOQ concentrations in dairy cows are positively related to insulin responsiveness and negatively associated with BCS prepartum (De Koster et al., 2017), indicating that greater BCS mitigates ADIPOQ secretion and potentially impairs insulin resistance. Although we did not measure ADIPOQ, a recent study from our laboratory reported that HBCS (BCS $\geq$ 3.75) compared with NBCS cows (BCS $\leq$ 3.25) had greater overall mRNA abundance of *ADIPOQ* in SAT from −10 to 20 d relative to calving (Alharthi et al., 2018). Thus, it could be possible that HBCS cows in the present study experienced hypoxia and alterations in ADIPOQ production and function. In support of this idea, feeding a high-concentrate diet leading to HBCS was accompanied by greater protein abundance of hypoxia-inducible factor 1α in SAT (Laubenthal et al., 2017). If HBCS cows experienced a state of hypoxia in SAT, this could partly explain the postpartal upregulation of *SLC38A1*, as reported in murine adipocytes (Horie et al., 2018).

The SLC38A1 protein is one of the major transporters of Gln (Mackenzie and Erickson, 2004). This AA not only serves as crucial oxidative fuel for immune cells such as macrophages and neutrophils (Newsholme et al., 2003), but attenuates inflammation in SAT caused by excessive fat deposition (Petrus et al., 2020). Obese individuals typically have lower Gln concentrations in SAT, which partly explains their susceptibility to macrophage infiltration and pro-inflammatory response (Petrus et al., 2020). It is well established that dairy cows experience an acute-phase response during early lactation (Moyes et al., 2010; Trevisi et al., 2012), which might be essential for normal immunometabolic functions. The fact that jugular Gln infusion (106 or 212 g/d) in peripartal cows without clinical signs of infectious or inflammatory diseases increased plasma concentrations of serum amyloid A and lipopolysaccharide-binding protein at d 7 after parturition and decreased concentrations of haptoglobin at d 14 and 21 postpartum. Jafari et al. (2006) suggested that postruminal Gln supply might contribute to improved immunometabolic status during early lactation; furthermore, lactating cows fed rumen-protected Gln (116 g/d) had greater plasma concentrations of IL-10, an anti-inflammatory cytokine (Caroprese et al., 2012), which underscored the role of this AA in the immune response.

As previously noted, an increase in macrophage infiltration in SAT during the periparturient period might exacerbate insulin resistance. Thus, along with lower activation of AKT (ratio of p-AKT to total AKT) at 7 d postpartum, the fact that HBCS cows experienced a more pronounced inflammatory response, compared with NBCS cows, particularly after parturition (Liang

et al., 2020), suggests that increased protein abundance of SLC38A1 in HBCS cows might have been associated with immune cell infiltration. Thus, we speculate that increased protein abundance of SLC38A1 is an adaptive response to counterbalance prolonged inflammatory status induced during periods of negative energy balance or as a result of excessive fat deposition prepartum.

mTOR Signaling

Differences in BCS had no effect on overall mRNA abundance of *EIF4EBP1* and *RPS6KB1* ($P = 0.92$; $P = 0.95$; Figure 3D and E), but a BCS × Day interaction was observed for *EIF4EBP1* due to its greater expression in HBCS cows at d 30 postpartum ($P = 0.04$; Figure 3D). This was similar to results observed in bovine skeletal muscle (Ghaffari et al., 2019b). Additionally, Ghaffari et al. (2019b) reported that mRNA abundance of proteins associated with protein degradation, such as ubiquitin-like modifier activating enzyme 1, ubiquitin-conjugating enzyme E2 G1, and atrogin-1, along with *mTOR*, was greater on d 21 postpartum in HBCS cows. Thus, their data support the idea that HBCS cows undergo a greater extent of body protein turnover during early lactation.

Amino acids are essential components of the protein synthesis cascade triggered by activation of the mTOR pathway (Laplante and Sabatini, 2009). In addition to the well-established role for Leu, Gln and Arg could activate mTOR (Bauchart-Thevret et al., 2010; van der Vos and Coffer, 2012). Beyond protein synthesis, data also indicate that deregulation of the mTOR signaling pathway in nonruminants is associated with obesity and metabolic disorders (Cai et al., 2016). Although in vivo and in vitro studies in bovines have provided evidence that AA supplementation regulates milk protein synthesis through activation of mTOR (Burgos et al., 2010; Rius et al., 2010; Appuhamy et al., 2012), less is known for adipose tissue. A previous study from our group revealed that enhanced postruminal supply of Met during the periparturient period enhanced mRNA abundance of AA transporters and protein abundance of p-mTOR in SAT (Liang et al., 2019), a response that correlated with lower oxidative stress and inflammation status (Batistel et al., 2018). Thus, we speculate that the greater mRNA abundance of *SLC7A1* and *SLC38A2* in HBCS cows might have contributed to the increased protein abundance of p-mTOR. Overall, data suggest that enhanced mRNA abundance of AA transporters and upregulated p-mTOR are part of an adaptive mechanism in HBCS cows to cope with peripartal oxidative stress and inflammation. Research with adipose tissue or cells exposed to oxidants could help shed light on this idea.

AA Metabolism, the Urea Cycle, and Inflammation

Main effects of BCS, day, and their interaction on mRNA abundance of key enzymes of the urea cycle are reported in Figure 7. Abundance of arginase 1 (*ARG1*) and argininosuccinate lyase (*ASL*) was greater overall in HBCS cows ($P = 0.05$; $P = 0.03$; Figure 7A and C), suggesting a greater degree of urea cycle activity in those cows. Although it is well established that the urea cycle in mammals is confined to the liver (Cynober et al., 1995), a previous study demonstrated that rat SAT contains a complete set of urea cycle enzymes: argininosuccinate synthase (*ASS1*), ASL, and ARG1 (Arriarán et al., 2015). Thus, available data suggest that AT plays a role in regulating Arg metabolism (Cynober et al., 1995), potentially via Cit synthesis (Arriarán et al., 2015). Greater overall mRNA abundance of *ARG1* and *ASL* in HBCS cows did not accompany greater plasma urea concentrations. Additionally, except for increased *ASS1* postpartum (Day, $P < 0.01$; Figure 7B), day relative to calving did not affect mRNA abundance of *ARG1* and *ASL* ($P = 0.22$; $P = 0.18$; Figure 7A and C), which is inconsistent with decreased plasma urea concentrations after parturition (Day, $P < 0.01$; Figure 2E). Thus, results suggest that the urea cycle in SAT might not make a great contribution to circulating levels of urea.

Arginase not only hydrolyzes Arg to Orn and urea but also regulates nitric oxide (NO) synthesis through competition with endothelial NO synthase for Arg (Durante, 2013). Activation of ARG and the reduction in NO synthesis can increase inflammation in nonruminants (Durante, 2013). In contrast, both in vivo and in vitro data from rats indicate that ARG inhibition reduced macrophage infiltration and pro-inflammatory cytokine production (Hu et al., 2015). Although speculative, the upregulation of the Arg transporter *SLC7A1* in HBCS cows might partly explain the increased mRNA abundance of *ASL* and *ARG1*, but also macrophage infiltration that could trigger a local inflammatory response. Thus, we speculate that an important role for a functional urea cycle in SAT is to control inflammation, especially in overconditioned cows. This idea is supported in part by the greater mRNA abundance of toll-like receptor 4 (*TLR4*) and *TLR9* in SAT of peripartal HBCS cows (BCS $\geq$ 3.75) reported by Alharthi et al. (2018).

Our group was the first to report the immune responsiveness of dairy cow SAT (Mukesh et al., 2010) and subsequently catalog differences in mRNA abun-

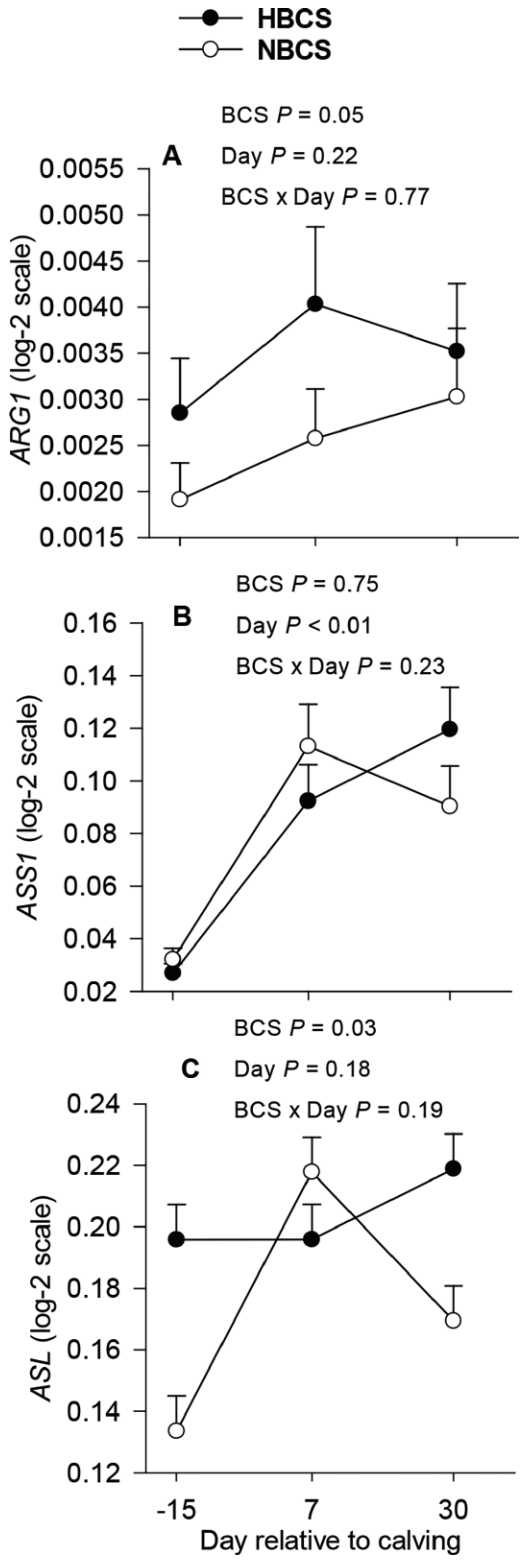

Figure 7. mRNA abundance of genes related to the urea cycle in subcutaneous adipose tissue of Holstein cows with prepartum (28 d before expected parturition) high (HBCS, BCS ≥3.5) and normal BCS (NBCS, BCS ≤3.17) at −15, 7, and 30 d relative to calving. Data are LSM, n = 11 cows per group, ± pooled SEM. *ASL* = argininosuccinate lyase; *ARG1* = arginase 1; *ASS1* = argininosuccinate synthase.

dance of immune-related genes in various fat depots in response to control or higher-energy diets (Ji et al., 2014b; Moisá et al., 2017; Minuti et al., 2020). More recently, a positive association between mRNA abundance of leptin, *IL-6*, and tumor necrosis factor in SAT with adipocyte size was reported (Depreester et al., 2018). In the context of inflammatory responsiveness of AT, such a relationship was particularly important because adipocyte size increases with BCS (De Koster et al., 2016). Thus, the greater macrophage counts in SAT from cows losing more body mass postpartum (Newman et al., 2019), along with the greater loss of BW and BCS we detected in HBCS cows, underscore the greater susceptibility to a chronic state of inflammation (Contreras et al., 2015). The exact molecular mechanisms whereby AA metabolism and urea cycle activity regulate SAT immune responsiveness remain to be established.

CONCLUSIONS

Overconditioning during the late prepartum period leads to lower activation of AKT (ratio of p-AKT to total AKT) and more pronounced lipolysis postpartum; however, adipose tissue in overconditioned cows might trigger compensatory mechanisms, leading to upregulation of AA transport and activation of mTOR signaling. Thus, beyond use of AA for protein synthesis, adipose tissue of overconditioned cows seems to utilize AA to alleviate negative effects caused by a prolonged inflammatory response. Urea cycle activity, in particular, seems to be important in the context of inflammation in overconditioned cows. Further research is warranted to ascertain the potential roles of AA, such as Met, Arg, Gln, and Glu, in SAT in the context of physiological challenges that lead to inflammation and oxidative stress.

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