Alterations in skeletal muscle abundance of protein turnover, stress, and antioxidant proteins during the periparturient period in dairy cows fed ethyl-cellulose rumen-protected methionine

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ABSTRACT

Skeletal muscle turnover helps support the physiological needs of dairy cows during the transition into lactation. We evaluated effects of feeding ethyl-cellulose rumen-protected methionine (RPM) during the periparturient period on abundance of proteins associated with transport AA and glucose, protein turnover, metabolism, and antioxidant pathways in skeletal muscle. Sixty multiparous Holstein cows were used in a block design and assigned to a control or RPM diet from −28 to 60 d in milk. The RPM was fed at a rate of 0.09% or 0.10% of dry matter intake (DMI) during the prepartal and postpartal periods to achieve a target Lys:Met ratio in the metabolizable protein of ~2.8:1. Muscle biopsies from the hind leg of 10 clinically healthy cows per diet collected at −21, 1, and 21 d relative to calving were used for western blotting of 38 target proteins. Statistical analysis was performed using the PROC MIXED statement of SAS version 9.4 (SAS Institute Inc.) with cow as random effect, whereas diet, time, and diet × time were the fixed effects. Diet × time tended to affect prepartum DMI, with RPM cows consuming 15.2 kg/d and controls 14.6 kg/d. However, diet had no effect on postpartum DMI (17.2 and 17.1 ± 0.4 kg/d for control and RPM, respectively). Diet or time did not affect the abundance of several AA transporters or the insulin-induced glucose transporter (SLC2A4). Among evaluated proteins, feeding RPM led to lower overall abundance of proteins associated with protein synthesis (phosphorylated EEF2, phosphorylated RPS6KB1), mTOR activation (RRAGA), proteasome degradation (UBA1), cellular stress responses (HSP70, phosphorylated MAPK3, phosphorylated EIF2A, ERK1/2), antioxidant response (GPX3), and de novo synthesis of phospholipids (PEMT). Regardless of diet, there was an increase in the abundance of the active form of the master regulator of protein synthesis phosphorylated MTOR and the growth-factor-induced serine/threonine kinase phosphorylated AKT1 and PIK3C3, whereas the abundance of a negative regulator of translation (phosphorylated EEF2K) decreased over time. Compared with d 1 after calving and regardless of diet, the abundance of proteins associated with endoplasmic reticulum stress (XBP1 spliced), cell growth and survival (phosphorylated MAPK3), inflammation (transcription factor p65), antioxidant responses (KEAP1), and circadian regulation (CLOCK, PER2) of oxidative metabolism was upregulated at d 21 relative to parturition. These responses coupled with the upregulation of transporters for Lys, Arg, and His (SLC7A1) and glutamate/aspartate (SLC1A3) over time were suggestive of dynamic adaptations in cellular functions. Overall, management approaches that could take advantage of this physiological plasticity may help cows make a smoother transition into lactation.

Key words: lactation, mechanistic target of rapamycin kinase, oxidative stress, proteasome

INTRODUCTION

Postpartal dairy cows often do not consume enough feed to support requirements for milk synthesis, which causes a state of negative energy and protein balance (NRC, 2001; Mann et al., 2016). To meet these nutrient requirements, cows in early lactation mobilize vast amounts of body reserves, including fat and muscle (Waltner et al., 1993; Phillips et al., 2003; Hatfield et
The degree of body weight loss during the periparturient period seems to be an intrinsic trait in high-yielding dairy cows (Zachut and Moallem, 2017), but overcatabolism of tissue can cause negative effects on health and performance (Kerwin et al., 2022). Muscle protein breakdown in cows seems to start prepartum, which is likely due to a gradual deficiency of AA supply (along with changes in the endocrine environment) as the rate of voluntary DMI decreases (Yang et al., 2020). Furthermore, during the peripartal period, an increase in plasma concentrations of fatty acids and reactive oxygen species (ROS) contributes to oxidative stress status and has a negative effect on both production performance and welfare.

Methionine typically is the first-limiting AA for lactating cows (NRC, 2001). Feeding rumen-protected methionine (RPM) to achieve a dietary Lys:Met ratio close to 2.8:1 in the MP during the peripartal period alleviated oxidative stress status and improved DMI and milk yield (Osorio et al., 2014; Batistel et al., 2017b). Inclusion of RPM in the diet altered the abundance of proteins associated with mechanistic target of rapamycin kinase (mTOR), insulin signaling, and 1-carbon metabolism in the liver of lactating cows (Coleman et al., 2022). In another study, Liang et al. (2019) demonstrated that RPM activated glutathione metabolism and the antioxidant NFE2L2 pathway in subcutaneous adipose tissue of peripartal cows. Thus, supplementation of RPM during the peripartal period could represent an effective dietary approach to diminish muscle protein breakdown and oxidative stress while providing extra Met to initiate translation.

The underlying mechanisms regulating protein turnover in skeletal muscle during the peripartal period seem to encompass alterations in protein synthesis along with upregulation of the mRNA and protein abundance of proteolytic pathway components (Ghaffari et al., 2019; Yang et al., 2020). In one of the first studies on this topic, Du et al. (2005) reported that feeding a nutrient-restricted diet to pregnant beef cows downregulated mTOR signaling and increased the amount of ubiquitinated proteins in skeletal muscle, both of which indicated a decrease in protein synthesis and accelerated protein degradation. Beyond these pathways, studies in nonruminants have highlighted the existence of regulatory proteins and signaling pathways within skeletal muscle that respond to nutrient supply or exogenous stressors (e.g., high fatty acid loads) to coordinate metabolic responses [circadian clock regulator (CLOCK)], antioxidant responses [NFE2 like bZIP transcription factor 2 (NFE2L2); tumor protein 53 (TP53)], and endoplasmic reticulum (ER) stress (Khapre et al., 2014; Beyfuss and Hood, 2018; Wible et al., 2018). Because these cellular pathways are likely important in the overall response of skeletal muscle to nutrition and the onset of parturition, an integrated evaluation of key components in these pathways is necessary to generate a more holistic view of the underlying mechanisms.

The specific objective of this study was to use skeletal muscle biopsy tissue from a subset of cows fed ethyl-cellulose RPM in one of our previous studies (Batistel et al., 2017b) to investigate protein abundance of selected targets associated with AA and glucose transport, insulin and growth-factor signaling, cellular metabolism, and antioxidant pathways during the peripartal period.

MATERIALS AND METHODS

Animal Housing and Care

All experimental procedures were performed according to approved protocols by the Institutional Animal Care and Use Committee at the University of Illinois (Urbana; protocol no. 14270). All cows were housed in a freestall barn equipped with a gate system (American Calan Inc.) during the prepartal period. They were then housed in tiestalls during the postpartal period. Cows were fed once daily (1300 h) at 120% of expected intake and milked 3 times daily (at 0600, 1400, and 2200 h).

Experimental Design and Diets

The present work used a subset of cows with a complete set of biopsies from the larger cohort used to determine production performance effects of RPM (Batistel et al., 2017b). Briefly, the entire cohort involved 60 multiparous Holstein Friesian cows from the University of Illinois Dairy Research Farm (Urbana, IL) arranged in a randomized complete block design experiment with 30 cows per treatment. Cows were blocked by expected parturition day, and the blocks were balanced by parity, previous 305-d milk yield, and BCS. Cows within each block were randomly assigned to 1 of the 2 treatments. Treatment diets were a basal control diet with no Met supplementation or the basal diet supplemented with ethyl-cellulose RPM (Mepron, Evonik Nutrition and Care GmbH). Ethyl-cellulose RPM was supplied from −28 to 60 d relative to parturition at a rate of 0.09 and 0.10% DMI of the previous day during the prepartal and postpartal periods, respectively. These target values were based on previous experiments demonstrating a benefit in terms of production performance and health.
of supplementing RPM to achieve a Lys:Met ratio close to 2.8:1 during the prepartal and postpartal periods.

**Sampling**

Per the approved protocol by the Institutional Animal Care and Use Committee (Urbana, IL), only 10 cows each in control and RPM groups could be subjected to needle biopsies of the semitendinosus muscle at −21, 1, and 21 d around parturition. This muscle was chosen because it experiences alterations in total CP content and AA profiles around parturition (Kuhla et al., 2011). Alternating sides of the animal were biopsied at each of these times after the morning milking. Briefly, the hair in a 20 × 20 cm² area was shaved close to the skin before application of Dermachlor 4% surgical scrub (Butler Animal Health Supply) solution for presurgical skin cleaning. Subsequently, a 5-mL lidocaine solution (2% Hydrochloride, 20 mg/mL; Agri Laboratories) was injected subcutaneously and intramuscularly and a 1-cm incision made approximately 10 min after anesthesia. Approximately 500 mg of muscle tissue was collected using a needle biopsy instrument (BARD Magnum, 14 gauge × 16 cm; C. R. Brad Inc.). Muscle tissue was snap-frozen in liquid nitrogen and then stored at −80°C until protein extraction. A similar approach for sample preparation and long-term storage has been used in previous peripartal cow studies (Webb et al., 2019; Yang et al., 2020). A surgical staple was applied to close the incision and removed at 6 d after biopsy, as appropriate. These cows were on their respective treatments for 28 ± 3 d before calving.

**Western Blotting**

The relative abundance of target proteins in skeletal muscle was detected using the same protocols published in our previous studies (Batistel et al., 2017a) and with antibodies validated in our laboratory (Elolimy et al., 2019; Hu et al., 2020). Briefly, 75 mg of muscle tissue were homogenized in T-PER tissue protein extraction reagent (cat. no. 78510; Thermo Fisher Scientific), which included an inhibitor cocktail (cat. no. 78440; Thermo Fisher Scientific). The lysates were centrifuged at 14,000 × g for 10 min at 4°C, and the supernatants were then harvested for measuring protein content using a BCA protein assay kit (cat. no. 23227; Thermo Fisher Scientific). Concentration of extracted protein was measured using a NanoDrop ND-1000 spectrophotometer instrument (Thermo Fisher Scientific). Extracted total protein from each sample was aliquoted into at least 3 cryovials, and each subsample was used only twice, if needed. The samples were diluted (5 μg/μL) and each diluted protein sample was divided into three 1.5-mL tubes and stored at −80°C until analysis. Before western blot analysis, samples were denatured at 95°C for 10 min after being diluted (3:1, vol/vol) with 4× Laemmli buffer (cat. no. 1610747; Bio-Rad).

The western blotting approach was a “2-step probing” (not stripped blotting) as follows. A total of 37.5 μg protein from each sample were separated using 4–20% Mini-PROTEAN TGX precast gels (cat. no. 4561096; Bio-Rad) and transferred onto a polyvinylidene difluoride membrane (cat. no. 1620261; Bio-Rad) using a Trans-Blot SD semi-dry electrophoretic transfer cell (cat. no. 1703940; Bio-Rad) for 25 min. After blocking with 5% nonfat dry milk in tris-buffered saline at room temperature for 2 h, the membrane was washed and incubated with primary antibodies listed in Supplemental Table S1 (https://doi.org/10.17632/vxvnb33ybd.1; Loor, 2023). The polyvinylidene difluoride membranes were then washed 6 times (5 min per time) and incubated with a secondary antibody for 1.5 h at room temperature before being washed again. Visualization of the target proteins was performed using the Clarity Western ECL Substrate (cat. no. 1705060; Bio-Rad). The enhanced chemiluminescence signals were recorded using an imaging system (ChemiDoc MP, Bio-Rad) and analyzed using Image Lab 3.0 software (Bio-Rad). The intensity of GAPDH was used to normalize the abundance of target proteins (Supplemental Figure S1; https://doi.org/10.17632/vxvnb33ybd.1; Loor, 2023).

**Preparation of mRNA and Real-Time Quantitative Reverse-Transcription PCR**

Approximately 50 mg of frozen muscle tissue were transferred to 1 mL of TRIzol reagent (Invitrogen, Thermo Fisher Scientific). RNA was extracted using standard procedures as described in our previous publications (Coleman et al., 2019). Concentration of extracted RNA was measured with a NanoDrop ND-1000 (Thermo Fisher Scientific). Quality of extracted RNA was analyzed in a Fragment Analyzer (Advanced Analytical Technologies Inc.). RNA samples had an RNA quality number ≥7.0 and were deemed suitable for mRNA abundance analysis. The high RNA quality number indicated that samples were viable in spite of the long-term storage at −80°C. For cDNA synthesis, 100 ng of RNA sample was reverse-transcribed following standard protocols in our laboratory (Coleman et al., 2019) and stored at −80°C until analysis.

Primers for *UBE2G1*, *UBE2G2*, *FBXO32*, *TRIM63* (Yang et al., 2020), and *4EBP1* (Bionaz and Loor, 2011) were obtained from published papers. Primers for *IGF1* were retrieved from the NCBI (https://www.ncbi.nlm.nih.gov/) and manufactured by Integrated DNA Technologies (https://www.idtdna
The MIXED procedure of SAS version 9.4 (SAS Institute Inc.) was used for repeated measures analysis using the following model:

\[ Y_{jl} = \mu + D_j + T_l + DT_{jl} + e_{jl}, \]

where \( Y_{jl} \) = dependent continuous variable, \( \mu \) = overall mean, \( D_j \) = fixed effect of diet (\( j \) = control vs. RPM), \( T_l \) = fixed effect of day (−21, 1, and 21 d), \( DT_{jl} \) = interaction between diet and day, and \( e_{jl} \) = residual error. The fixed effects in the model were diet and time, and the random effect was cow nested within diet. Data for DMI from the cows in this study were analyzed separately for the prepartal (−21 to −1 DIM) and the postpartal (1–30 DIM) periods. Milk yield for these cows encompassed d 1 through 30 of the postpartum period. The degrees of freedom were corrected using the Kenward-Roger method, which yields more precise and efficient estimates of the fixed effects in experiments with a moderate sample size. 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.

### Results

#### Milk Yield and DMI

An interaction of diet × time (\( P = 0.10 \)) for DMI tended to occur in the prepartal period, where controls averaged 14.6 ± 0.4 kg/d and RPM 15.2 ± 0.4 kg/d (Figure 1). However, neither the interaction (\( P = 0.96 \)) nor the main effect of diet (\( P = 0.86 \)) were significant for postpartal DMI. Milk yield during the first 30 DIM also did not differ between diets (interaction \( P = 0.47 \), treatment \( P = 0.64 \)). Milk yield and DMI both had a significant (\( P < 0.001 \)) effect of time (Figure 1).

#### Protein Abundance in Skeletal Muscle

##### Amino Acid and Glucose Transport.

Diet had no effect on abundance of proteins associated with insulin-induced glucose transport (solute carrier family 2 member 4, SLC2A4), but the abundance of sodium-dependent glutamate/aspartate transporter (solute carrier family 1 member 3, SLC1A3) and cationic AA transport (solute carrier family 7 member 1, SLC7A1) changed over time (Figure 2). Compared with the first day relative to calving (0.64 for SLC1A3 and 1.47 for SLC7A1), expression of SLC1A3 (\( P < 0.05 \); Figure 2) and SLC7A1 (\( P < 0.01 \); Figure 2) was higher at 21 d (0.93 for SLC1A3 and 2.47 for SLC7A1). No effects were noted for the abundance of the AA transporters solute carrier family 1 member 5 (sodium-dependent neutral AA transport, SLC1A5), solute carrier family 38 member 1 (glutamine transporter, SLC38A1), or solute carrier family 7 member 11 (anionic AA transporter, SLC7A11; Figure 2).

##### Protein Synthesis via mTOR.

Among detected proteins involved in the control of protein synthesis, abundance of phosphorylated eukaryotic translation elongation factor 2 (pEEF2; 1.45 for RPM vs. 2.28 for control) and ribosomal protein S6 kinase B1 (RPS6KB1) was overall lower (0.84 for RPM vs. 1.37 for control; \( P < 0.05 \)) in RPM cows (Figure 3). In contrast, regardless of diet, the abundance of phosphorylated proteins associated with the control of protein synthesis via extracellular signals such as phosphorylated AKT serine/threonine kinase 1 (pAKT1), phosphorylated mechanistic target of rapamycin kinase (pMTOR), phosphorylated eukaryotic elongation factor 2 kinase...
(pEEF2K), and phosphatidylinositol 3-kinase catalytic subunit type 3 (PIK3C3) was affected by day relative to calving. Compared with d 1 relative to calving (0.98 for pAKT1 and 0.67 for pMTOR), a greater abundance of pAKT1 ($P = 0.06$) and pMTOR ($P < 0.05$) was detected at 21 d (1.50 for pAKT1 and 0.95 for pMTOR, respectively). Similarly, PIK3C3 increased markedly ($P < 0.01$) at 21 d relative to parturition (1.0 at 1 d and 2.4 at 21 d). In contrast, compared with −21 d prepartum (0.60), pEEF2K abundance decreased gradually and was overall lower ($P < 0.05$; Figure 3) at 21 d (0.27).

**Amino Acid Sensing.** Among proteins associated with intracellular AA sensing and mTOR activity, abundance of Ras related GTP binding A (RRAGA) was the only one with an overall diet effect (Figure 4), with clear downregulation in RPM versus control cows (1.29 for mTOR vs. 2.33 for control), especially at d 1 and 21 relative to calving. There was an overall time effect for the abundance of meiosis regulator for oocyte development (MIOS) and TSC complex subunit 2 (TSC2) due to upregulation at 21 d relative to −21 d around to calving (MIOS, 1.77 at 21 d and 1.18 at −21 d; TSC2, 1.78 at 21 d and 1.04 −21 d; Figure 4). There were no diet or time effects for the abundance of cytosolic arginine sensor for mTORC1 subunit 1 (CATOR1).

**Circadian Regulators Associated with mTOR.** Among the components of the circadian oscillator pathway measured, the abundance of basic helix-loop-helix ARNT like 1 (BMAL1), which forms a heterodimer with CLOCK, tended to be greater overall ($P = 0.09$) in cows fed RPM (1.10 for RPM vs. 0.61 for control), especially due to responses at d −21 and 1 around calving. The abundance of period 2 (PER2), which is activated by circadian regulator (CLOCK)/BMAL1, decreased between d −21 and 1 (2.05 at −21 vs. 0.90 at 1 d) and then increased markedly at 21 d (2.18 at 21 d and 0.90 at 1 d) relative to calving ($P < 0.001$; Figure 5). Abundance of CLOCK tended ($P = 0.11$; Figure 5) to increase by d 21 compared with d 1 (1.42 at 21 d and 0.90 at 1 d), while abundance of cryptochrome circadian regulator 1 (CRY1) was not affected by diet or time.

**Ubiquitination and Protein Degradation.** Among proteins involved in protein degradation via ubiquitination, the abundance of ubiquitin like modifier activating enzyme 1 (UBA1) was overall lower (0.89 for RPM and 1.34 for control), and that of E3 ubiquitin protein ligase (MDM2) was overall greater (0.99 for RPM and 0.53 for control; $P < 0.05$) in RPM cows (Figure 6). Regardless of diet, compared with −21 and 1 d relative to calving (1.01 at −21 and 0.94 at 1 d),

**Figure 1.** Prepartal and postpartal DMI and milk production in dairy cows (n = 10/diet) fed a basal control diet or the basal diet plus ethyl-cellulose rumen-protected methionine (RPM) from −28 to 30 d around calving. Data for DMI prepartum used for statistical analysis encompassed the period of −21 d to −1 d around calving. Error bars represent SEM.
Figure 2. Abundance of proteins associated with AA and glucose transport in skeletal muscle of dairy cows (n = 10/diet) fed a basal control diet or the basal diet plus ethyl-cellulose rumen-protected methionine (RPM) from −28 to 30 d around calving: SLC1A5 (A), neutral AA transporter (phenylalanine, tyrosine, tryptophan, threonine, methionine, valine, isoleucine, leucine, and histidine), solute carrier family 1 member 5; SLC38A1 (B), glutamine transporter, solute carrier family 38 member 1; SLC7A1 (C), histidine transporter, cationic AA transporter (arginine, lysine, ornithine); SLC7A11 (D), anionic AA transporter (cysteine, glutamate), solute carrier family 7 member 11; SLC1A3 (E), sodium-dependent glutamate/aspartate transporter, solute carrier family 1 member 3; and SLC2A4 (F), insulin-induced glucose transporter, solute carrier family 2 member 4. Skeletal muscle biopsies were harvested at −21, 1, and 21 d around calving. Error bars represent SEM.
Figure 3. Abundance of proteins associated with insulin- and growth-factor–induced protein synthesis in skeletal muscle of dairy cows (n = 10/diet) fed a basal control diet or the basal diet plus ethyl-cellulose rumen-protected methionine (RPM) from −28 to 30 d around calving: pMTOR (A), phosphorylated mechanistic target of rapamycin kinase; pAKT1 (B), phosphorylated AKT serine/threonine kinase 1; pEEF2K (C), phosphorylated eukaryotic elongation factor 2 kinase; pEEF2 (D), phosphorylated eukaryotic translation elongation factor 2; PIK3C3 (E), phosphatidylinositol 3-kinase catalytic subunit type 3; and RPS6KB1 (F), ribosomal protein S6 kinase B1. Skeletal muscle biopsies were harvested at −21, 1, and 21 d around calving. Error bars represent SEM.
UBA1 abundance was higher ($P = 0.04$; Figure 6) at 21 d postpartum (1.38).

**Stress and Antioxidant Responses.** Among the stress-responsive phosphorylated proteins studied, phosphorylated mitogen-activated protein kinase 3 (pMAPK3; 2.07 for RPM vs. 3.26 for control) and phosphorylated eukaryotic translation initiation factor 2A (pEIF2A; 0.96 for RPM vs. 1.36 for control) were overall downregulated ($P < 0.05$) when cows were fed RPM (Figure 7). Compared with d 1 relative to calving (0.93), a higher ($P < 0.05$; Figure 7) abundance of X-box-binding protein (XBP1spliced) was detected at 21 d (1.57). Compared with other days, and regardless of diet, pMAPK3 was downregulated ($P < 0.05$) and pEIF2A upregulated ($P < 0.05$) at 1 d relative to calving. The abundance of the extracellular response

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**Figure 4.** Abundance of proteins associated with AA sensing in skeletal muscle of dairy cows (n = 10/diet) fed a basal control diet or the basal diet plus ethyl-cellulose rumen-protected methionine (RPM) from −28 to 30 d around calving: MIOS (A), meiosis regulator for oocyte development; RRAGA (B), Ras related GTP binding A; CASTOR1 (C), cytosolic arginine sensor for mTORC1 subunit 1; and TSC2 (D), TSC complex subunit 2. Skeletal muscle biopsies were harvested at −21, 1, and 21 d around calving. Error bars represent SEM.
kinase (ERK1/2) was overall lower ($P = 0.05$; Figure 7) in response to feeding RPM. The proinflammatory protein RELA proto-oncogene, NF-kB subunit (RELA) had an overall time effect ($P = 0.05$) due to upregulation at 21 d versus 1 d (2.19 vs. 1.29).

Among the antioxidant response proteins studied, the abundance of glutathione peroxidase 3 (GPX3) was lower overall (1.41 vs. 2.10; $P = 0.06$; Figure 8) and phosphorylated NFE2 like bZIP transcription factor 2 (pNFE2L2) tended ($P = 0.11$) to be lower (0.26 vs. 0.49) in response to feeding RPM (Figure 8). Although diet had no effect ($P > 0.05$) on the abundance of kelch like ECH associated protein 1 (KEAP1) and heme oxygenase 1 (HMOX1; both associated with pNFE2L2), a greater abundance was detected for KEAP1 at 21 d (0.99) versus 1 d relative to calving (0.51).

Figure 5. Abundance of proteins associated with circadian regulation of metabolism in skeletal muscle of dairy cows ($n = 10$/diet) fed a basal control diet or the basal diet plus ethyl-cellulose rumen-protected methionine (RPM) from −28 to 30 d around calving: CLOCK (A), clock circadian regulator; BMAL1 (B), basic helix-loop-helix ARNT like 1; and CRY1 (D), cryptochrome circadian regulator 1. Skeletal muscle biopsies were harvested at −21, 1, and 21 d around calving. Error bars represent SEM.
Phospholipid Synthesis and Methylation.
Abundance of phosphatidylethanolamine N-methyltransferase (PEMT), a protein involved in phosphatidylcholine synthesis, was overall lower (\(P < 0.001\)) in cows fed RPM (2.15 for RPM vs. 3.26 for control; Figure 9). No effects were noted for the abundance of methionine adenosyltransferase 1A (MAT1A) responsible for synthesizing S-adenosylmethionine, a key methyl donor (Figure 9).

Carnitine Synthesis, Intracellular Lipolysis, and Energy.
No effects were noted for the abundance of gamma-butyrobetaine hydroxylase 1 (BBOX1), which catalyzes the rate-limiting step in de novo synthesis of carnitine (Figure 10). Similarly, no effects were noted for the abundance of adipose triglyceride lipase, which is responsible for the control of basal lipolysis of intracellular triglyceride. The intracellular ATP sensor phosphorylated protein kinase AMP-activated catalytic subunit α 1 (pAMPK) was not affected by diet or time.

Abundance of mRNA
Because no validated antibodies are available for bovine (to our knowledge), we used RT-PCR to assess the abundance of key components of muscle protein turnover. There was diet \(\times\) time interaction for the mRNA abundance of the muscle-specific ligase TRIM63 (also known as MuRF-1; \(P = 0.05\)) and IGF1 (\(P < 0.05\); Figure 11). Compared with control (1.22), cows fed RPM had greater (\(P < 0.01\)) mRNA abundance of TRIM63 (2.24) at 1 d after calving (Figure 11). However, cows fed RPM had lower mRNA abundance of IGF1 (1.13 for RPM vs. 2.11 for control; \(P < 0.01\)) relative to control cows at 21 d after calving (Figure 11). Cows fed RPM also had greater (\(P = 0.05\); Figure 11) mRNA abundance of 4EBP1 compared with control. Regardless of diet, the mRNA abundance of 4EBP1 was upregulated (\(P < 0.001\); Figure 11) at 1 d relative to calving. No diet or time effects were noted for the mRNA abundance of genes encoding proteins associated with proteasome activity (UBE2G1, UBE2G2, and FBXO32; Figure 12).

DISCUSSION
Amino Acid and Glucose Transport
The transition from late gestation to early lactation is marked by an almost twofold increase in AA requirements along with a 3-fold increase in energy demands (Bell et al., 2000). As a result, cellular mobilization and mitochondrial metabolic output in muscle are increased (Phillips et al., 2003; Hatfield et al., 2022; Sadri et al., 2022). The lack of statistical effect on proteins involved in AA and insulin-induced glucose transport between groups agrees with the similar DMI and milk yield, thus suggesting no overt shortfall in the availability of these nutrients within muscle. Holistically, the lack of change in SLC2A4 across the peripartal period might represent a mechanism to spare glucose for use by the fetus (during pregnancy) or the mammary gland after calving. Based on its established role in nonruminant

Figure 6. Abundance of proteins associated with protein degradation in skeletal muscle of dairy cows (\(n = 10/diet\)) fed a basal control diet or the basal diet plus ethyl-cellulose rumen-protected methionine (RPM) from −28 to 30 d around calving: UBA1 (A), ubiquitin like modifier activating enzyme 1; and MDM2 (B), MDM2 proto-oncogene (E3 ubiquitin ligase). Skeletal muscle biopsies were harvested at −21, 1, and 21 d around calving. Error bars represent SEM.
Figure 7. Abundance of proteins associated with the stress response in skeletal muscle of dairy cows (n = 10/diet) fed a basal control diet or the basal diet plus ethyl-cellulose rumen-protected methionine (RPM) from −28 to 30 d around calving: HSP70 (A), heat shock protein family A (Hsp70); XBP1spliced (B), X-box-binding protein 1; pEIF2A (C), phosphorylated eukaryotic translation initiation factor 2A; pMAPK3 (D), phosphorylated mitogen-activated protein kinase 3; ERK1/2 (E), extracellular signal-regulated kinase; and RELA (F), RELA proto-oncogene, NF-kB subunit. Skeletal muscle biopsies were harvested at −21, 1, and 21 d around calving. Error bars represent SEM.
Figure 8. Abundance of proteins associated with antioxidant responses in skeletal muscle of dairy cows (n = 10/diet) fed a basal control diet or the basal diet plus ethyl-cellulose rumen-protected methionine (RPM) from −28 to 30 d around calving: GPX1 (A), glutathione peroxidase 1; GPX3 (B), glutathione peroxidase 3; KEAP1 (C), kelch like ECH associated protein 1; HMOX1 (D), heme oxygenase 1; and pNFE2L2 (E), phosphorylated NFE2 like bZIP transcription factor 2. Skeletal muscle biopsies were harvested at −21, 1, and 21 d around calving. Error bars represent SEM.
models, it is possible that the circadian regulator BMAL1 exerts some degree of control over the local insulin response because silencing of this protein in mouse skeletal muscle impaired glucose uptake and oxidation (Khapre et al., 2014). Although further research could answer specific questions about mechanisms, the marked upregulation of CLOCK and its target PER2

\[ \text{Figure 9. Abundance of proteins associated with phospholipid synthesis and global methylation in skeletal muscle of dairy cows (n = 10/diet) fed a basal control diet or the basal diet plus ethyl-cellulose rumen-protected methionine (RPM) from -28 to 30 d around calving: PEMT (A), phosphatidylethanolamine N-methyltransferase; and MAT1A (B), methionine adenosyltransferase 1A. Skeletal muscle biopsies were harvested at -21, 1, and 21 d around calving. Error bars represent SEM.} \]

\[ \text{Figure 10. Abundance of proteins associated with carnitine synthesis, basal triacylglycerol lipolysis, and intracellular energy sensing in skeletal muscle of dairy cows (n = 10/diet) fed a basal control diet or the basal diet plus ethyl-cellulose rumen-protected methionine (RPM) from -28 to 30 d around calving: BBOX1 (A), gamma-butyrobetaine hydroxylase 1; ATGL (B), adipose triglyceride lipase (patatin like phospholipase domain containing 2); and pAMPK (C), phosphorylated protein kinase AMP-activated catalytic subunit \( \alpha 1 \). Skeletal muscle biopsies were harvested at -21, 1, and 21 d around calving. Error bars represent SEM.} \]
Figure 11. mRNA abundance of genes associated with proteolysis and growth factor-induced protein synthesis in skeletal muscle of dairy cows (n = 10/diet) fed a basal control diet or the basal diet plus ethylcellulose rumen-protected methionine (RPM) from −28 to 30 d around calving: TRIM63 (A; also known as MuRF-1), IGF1 (B), and 4EBP1 (C). Error bars represent SEM. *Diet × time = P < 0.05.

Figure 12. mRNA abundance of genes associated with ubiquitination and protein degradation in skeletal muscle of dairy cows (n = 10/diet) fed a basal control diet or the basal diet plus ethylcellulose rumen-protected methionine (RPM) from −28 to 30 d around calving: UBE2G1 (A), UBE2G2 (B), and FBX032 (C). Skeletal muscle biopsies were harvested at −21, 1, and 21 d around calving. Error bars represent SEM.
at 21 relative to 1 d after calving was indicative of potential biological roles within muscle. For example, upregulation of CLOCK at 21 d might help alleviate the negative effect of molecules such as free fatty acids that can impair insulin sensitivity (Khapre et al., 2014). A lack of change in BMAL1 during the transition period also could favor activation of the mTOR pathway in muscle (Khapre et al., 2014) and help maintain its protein synthetic capacity.

The higher abundance at 21 versus 1 d postpartum of proteins (SLC1A3 and SLC7A1) that regulate the sodium-dependent glutamate/aspartate and cationic AA (Arg, Lys, and Orn) transport suggested some degree of plasticity of skeletal muscle for uptake or release of AA (i.e., “bidirectional flux”; Nicklin et al., 2009). Physiologically, such a response could help furnish essential and nonessential AA to the mammary gland but also allow myocytes to use circulating AA for protein synthesis, oxidation (e.g., in the Krebs cycle), or production of molecules such as nitric oxide (e.g., Arg uptake via SLC7A1). For example, the latter was a response observed in endothelial cells overexpressing SLC7A1 (Cui et al., 2011), whereas in colonic cells overexpressing SLC7A1, an accumulation of Arg that increased cell growth was observed (Lu et al., 2013). Clearly, the functional outcomes from the observed molecular responses might also depend on the degree of negative energy balance driven in part by the ability of the cow to reach optimal levels of DMI.

**Protein Synthesis and Degradation**

Translation is regulated at the initiation and elongation steps, with EEF2 playing an important role during peptide elongation (Hizli et al., 2013; Dinavahi et al., 2022). One mechanism that inhibits elongation is the phosphorylation of EEF2 by EEF2 kinase (EEF2K). Thus, the lower overall abundance of pEEF2 in RPM cows indicated a greater degree of protein elongation in myocytes compared with controls. The activity of EEF2K is regulated by multiple upstream signaling cascades through its phosphorylation (Huber-Keener et al., 2012). Regardless of diet, the linear decrease of pEEF2K abundance from −21 to 21 d relative to calving suggested that overall EEF2K activity was downregulated, which would have allowed for an increase in protein synthesis between late gestation and early lactation.

The increase of pAKT1, pMTOR, and PIK3C3 abundance from 1 to 21 d relative to calving suggested that the gradual increase in DMI regardless of diet observed in this subset of cows and also the full cohort (Batistel et al., 2017b) might have helped maintain some degree of tissue sensitivity to insulin or growth factors that are important in terms of AA utilization for protein synthesis. The upregulation of mRNA for IGF1 after calving regardless of diet provides some support for this idea. In addition, the lack of robust change in total AA content in muscle tissue and the modest decrease in total tissue protein content in the first 3 to 4 wk after calving (Kuhla et al., 2011) emphasize the presence of cellular mechanisms to counteract the catabolic state of early lactation.

Although diet did not affect the overall abundance of pAKT1 and pMTOR, the lower overall abundance of pEEF2, pRPS6KB1, and RRAGA in RPM cows suggested that their upregulation was not needed to stimulate translation. The lower overall UBA1 in cows fed RPM suggested that proteasome activity (and protein degradation) was more active in control cows. Small increases in proteasome activity and total ubiquitin protein in external oblique muscle were detected between −28 d and 21 d relative to parturition in dairy cows (Mann et al., 2016). Whether proteasome activity differs among muscle types in ruminants is unclear. However, such a scenario might explain the greater RRAGA abundance in control cows, a GTPase that recruits mTOR onto the lysosomal surface where it enhances mTOR activity leading to phosphorylation of RPS6KB1 and protein synthesis (Takahara et al., 2020).

Since the abundance of CASTOR1 (an Arg sensor and activator of mTOR), TSC2 (an Arg sensor that inhibits mTOR), and MIOS (an indirect activator of mTOR) was unaffected by diet, it is possible that regulation of protein synthesis via mTOR in skeletal muscle during early lactation (i.e., negative energy balance) is via the activity of RRAGA. Since UBA1 triggers the first step of the ubiquitin-proteasome pathway (i.e., it is classified as an E1 ligase), which also can induce apoptosis (Fu et al., 2015; Groen and Gillingwater, 2015), dietary treatments such as RPM that alter the abundance of these regulatory proteins might be helpful in terms of controlling skeletal muscle homeostasis around calving.

The loss of body protein during early lactation is due to increased skeletal muscle degradation, decreased protein synthesis, or both (Bell et al., 2000). Foletta et al. (2011) in a review of published studies with nonruminant species proposed that FBXO32 (also called atrogin-1) may be involved in regulation of the substrate targets affecting protein synthesis and muscle growth, whereas TRIM63 may play a role in the control of protein degradation and likely contributes to skeletal muscle metabolism. Thus, the unchanged mRNA abundance of FBXO32 in the present study seems to suggest that (1) it does not play a similar role in ruminants or (2) its function in ruminants might be controlled at the posttranscriptional level. However, the fact that FBXO32 protein abundance remained unchanged in...
skeletal muscle between −28 d and 21 d relative to parturition suggests that it may not play a crucial role in dairy cows (Mann et al., 2016). At the mRNA level, our data differed from the study of Yang et al. (2020) in which greater abundance of FBXO32 mRNA in skeletal muscle was detected at 21 d from calving in cows fed lipid as CLA versus saturated lipid starting on d 1 postpartum. Such a response was not entirely surprising because feeding CLA led to consistently lower DMI, which might have triggered catabolism of muscle to help sustain the AA needs of the lactating mammary gland.

Our finding that abundance of TRIM63 mRNA was 1.39-fold greater in RPM compared with control cows on d 1 relative to calving likely resulted from the tendency for greater DMI as parturition approached and, thus, the total postprandial supply of AA in this group (Batistel et al., 2017b). Since this effect of RPM was only transient, and UBA1 protein abundance was lower, it is unlikely that transcription of TRIM63 was associated with the ubiquitin-proteasome system in muscle. In fact, previous studies reported either a gradual decrease in TRIM63 protein abundance in skeletal muscle from −28 to 21 d around calving or no change (Mann et al., 2016; Yang et al., 2020) despite a modest increase in proteasome activity. Yang et al. (2020) reported that TRIM63 mRNA in skeletal muscle was markedly upregulated at d 1 after calving relative to d −21 and then decreased gradually through d 21. These responses agree with the pattern of expression we observed in the present study.

Given the catabolic state of the cow after parturition, reported changes in proteasome activity in skeletal muscle at the mRNA and protein level are not surprising. It is noteworthy that, in spite of moderately higher proteasome activity, the increase of pM TOR protein as lactation progresses (present study; Yang et al., 2020) may represent a feedback mechanism in the myocyte that helps prevent excessive protein degradation during early lactation. Such a response could be important not only in the context of structural proteins and metabolic enzymes but also antioxidant enzymes such as GPX whose abundance is closely regulated via ubiquitination (Zhu et al., 2022).

During the fed state, insulin and AA (in particular, branched-chain AA) mediate the stimulation of protein synthesis in muscle of nonruminant and nonlactating species (Takahara et al., 2020). The mTOR pathway integrates AA and insulin signals through multiple mechanisms including 4EBP1, and previous studies reported marked upregulation of 4EBP1 mRNA in muscle soon after calving, similar to what we observed (Yang et al., 2020). The greater overall abundance of 4EBP1 mRNA in cows fed RPM could be taken as another indication that protein synthesis was greater in these animals (Velloso, 2008; Glass, 2010). Together, the upregulation of mTOR, pAKT1, and especially PIK3C3 at 21 d after calving was highly suggestive of skeletal muscle retaining the ability to accrete muscle protein even during a period of negative energy and protein balance.

Stress and Antioxidant Responses

The upregulation of several genes associated with fatty acid oxidation in skeletal muscle after calving emphasized that myocytes readily use fatty acids to generate ATP (Schaff et al., 2013), the source of fatty acids being primarily the intracellular lipolysis of triglycerides (Kuhla et al., 2011). As such, the production of ROS might be a continuous process and likely accounts for the reported increase in skeletal muscle oxidative damage in early lactation cows (Favorit et al., 2021). In the present study, the similar abundance of BBOX1 (rate-limiting enzyme for endogenous carnitine synthesis) and adipose triglyceride lipase (regulator of basal triglyceride lipolysis) regardless of diet and time suggested that fatty acid oxidation within myocytes might be an active process independent of rate of DMI or physiological state. Thus, antioxidant mechanisms within myocytes might be essential to counterbalance production of ROS especially during early lactation when GPX activity decreases relative to the prepartal period (Favorit et al., 2021). Although we did not detect statistical differences in the abundance of GPX1 due to diet or time, the greater overall abundance of GPX3 when feeding the control diet might have played a protective role against excessive oxidative damage of myocytes (Favorit et al., 2021). This idea agrees with the greater UBA1 and lower MDM2 in cows fed the control diet and the lack of effect on FBXO32, which is known to enhance ubiquitination of GPX (Zhu et al., 2022).

The role of MDM2 in the response of muscle to oxidative stress is well-known in nonruminants, where its specific function is to ubiquitinate TP53 (key regulator of antioxidant responses) in the cytosol, tag it for degradation in the proteasome, and maintain it at low levels (Beyfuss and Hood, 2018). Activation of TP53 in muscle by oxidative stress or via activation of pMAPK3 triggers several signaling cascades, one of which leads to inhibition of ERK1/2 signaling (Beyfuss and Hood, 2018). Thus, although not measured in the present study, the combined profiles of these proteins along with those of pEIF2A and HSP70 was highly suggestive of a more pronounced oxidative stress state in muscle of control cows. Plasma biomarker data from cows in the present study confirmed that feeding RPM led to...
a reduction in oxidative stress and inflammation (Bastet et al., 2018). Previous studies reported that cows experiencing a greater state of oxidative stress (based on systemic biomarkers) had a greater abundance of proteins involved in the ubiquitin-proteasome pathway in skeletal muscle (Ghaaffari et al., 2019). Although not affected by diet, the upregulation of XBP1spliced and RELA between 1 and 21 d relative to parturition was indicative of an increase in ER stress and inflammation within muscle (El Golli-Bennour and Bacha, 2011). Whether these responses reflected infiltration of immune and proinflammatory cells or arose from increased tissue remodeling induced by the onset of lactation could not be discerned.

The KEAP1-NFE2L2 pathway constitutes the main protective mechanism used by cells in response to oxidative and electrophilic stresses (Bellezza et al., 2018). Under normal physiological conditions, NFE2L2 protein (antioxidant transcription factor) is maintained at a low level in the cytosol due to KEAP1-mediated proteasome-dependent degradation. Transcriptional control of NFE2L2 is also important, at least in nonruminants, and it involves the activity of CLOCK/BMAL1 (Wible et al., 2018). In response to stress inducers, the constitutive degradation of NFE2L2 is inhibited and allows NFE2L2 to accumulate within the cytosol, translocate to the nucleus, and regulate cytoprotective gene expression, including GPX (Baird and Yamamoto, 2020) and the core CLOCK repressor CRY1 (Wible et al., 2018). The latter emphasized the linkage between oxidative metabolism, endogenous oxidant signals, and circadian control of metabolism. An activation of the NFE2L2 pathway has been reported when proteasome activity is reduced, especially in tissues with high oxidative capacity, such as muscle (Dreger et al., 2009; Chen et al., 2022).

In the present study, the fact that pNFE2L2 tended to be greater in control versus RPM cows suggested that myocytes in these cows might have been exposed to a greater load of oxidative inducers. Such an effect would help explain the greater overall abundance of GPX3 in control cows. Considering the abundance of indicators of proteasome activity (e.g., UBA1) in control cows, it could be possible that, unlike nonruminants, greater proteasome activity is one trigger of NFE2L2 activation in dairy cows exposed to the stress of early lactation. A role for CRY1 in this regulation cannot be discounted because the pattern of abundance for NFE2L2 and CRY1 was similar over time. When assessing the longitudinal profile of KEAP1 regardless of diet, it is apparent that the regulation of the NFE2L2 pathway is dynamic and may be responsive to nutrients (e.g., methionine) that have the ability to increase the synthesis of antioxidants such as glutathione (Coleman et al., 2021). Given recent reports of substantial oxidative damage and reduced GPX activity in muscle during early lactation (Favorit et al., 2021), “feeding” this pathway might be an important target from a nutritional standpoint.

**Phospholipid Synthesis**

The difference in abundance of PEMT between cows fed RPM and the control diet was among the most consistent responses across the time points evaluated. This enzyme catalyzes the synthesis of phosphatidylcholine from phosphatidylethanolamine and S-adenosylmethionine generated in the Met cycle via MAT1A (Coleman et al., 2021). Because cell membrane phospholipids are integral to the proper functioning and stability of membrane-bound transporters (Verkerke et al., 2019), ER and mitochondrial function (Lagace and Ridgway, 2013), and cytosolic lipid droplet synthesis (Vance, 2013), these data suggest that feeding RPM by increasing Met supply affected all of these processes.

Although we lack other data to help explain the physiological meaning of differences in PEMT, in nonruminant knockout models, the lack of this protein is associated with increased basal oxidative muscle fiber oxygen consumption, metabolic rate, and ATP synthesis without alterations in mitochondrial morphology (Verkerke et al., 2019). Mechanistically, this effect is due to decreased sarcoplasmic reticulum Ca\(^{2+}\)-ATPase transport efficiency (Ca\(^{2+}\) uptake and ATP hydrolysis) without altering mitochondrial uncoupling. It remains to be determined whether energy metabolism in skeletal muscle is an important role for PEMT around parturition, especially because of recent data demonstrating adaptations in oxidative metabolism and antioxidant responses in muscle tissue of peripartal dairy cows (Favorit et al., 2021).

**CONCLUSIONS**

Skeletal muscle contains several proteins associated not only with AA use and protein turnover but also with cellular oxidation, stress, and antioxidant mechanisms. Some of these processes are dynamic around parturition and might be responsive to differences in DMI, which in the larger cohort of cows was greater with RPM, or directly by the supply of Met (e.g., as a source of methyl donors for phospholipid or lipid droplet synthesis). At a molecular level, the skeletal muscle retains some ability to respond to anabolic signals around parturition. It remains to be determined to what extent
the postruminal supply of specific nutrients can affect myocyte function during the transition period.

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