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Tissue mobilization to meet the nutritional requirements of a growing gravid uterus and strategies to mitigate its intensity in dairy cows

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Abstract The study investigated the effects of different diets on late-pregnant Holstein cows. Twenty-one multiparous cows were grouped into three dietary treatments: a control diet, a diet with rumen-protected amino acids (methionine and lysine; PAA), and a high-crude protein (High-CP) diet containing plant source proteins. The cows were transferred to individual stalls 28 days before calving and remained until parturition. Weekly measurements included the body weight (BW), body condition score (BCS), and back fat and eye muscle depths. Blood and urine samples were taken for analysis of β-hydroxy butyrate (βHB), non-esterified fatty acid (NEFA), cholesterol, and nitrogenous compounds. Colostrum and placental attributes were collected at calving. Results showed a 25% decrease in dry matter intake near calving, with stable BW and BCS. The cows that fed on High-CP, had the highest back fat and eye muscle depth. Blood metabolite analysis revealed decreasing anabolic markers [albumin, plasma urea nitrogen (PUN), cholesterol] and increasing catabolic markers (creatinine, βHB, NEFA) toward calving. Plasma 3-methylhistidine (3-MH) levels rose, indicating muscle mobilization. Urinary excretion of allantoin, total urea nitrogen, and uric acid decreased significantly ($P<0.001$), reflecting increased nitrogen demand. The cows that were fed with High-CP exhibited higher fecal nitrogen excretion, while the cows on PAA had lower metabolic fecal nitrogen (MFN) levels. The calf BW was highest in the PAA group, and their colostrum showed higher protein content and lower freezing points. This study highlights dynamic metabolic shifts in dairy cows during late pregnancy, with significant muscle and fat mobilization despite stable body metrics. High dietary CP could not prevent muscle mobilization, suggesting the need for adaptive feeding strategies. The results underline the importance of dietary supplementation, particularly PAA, to meet the heightened nutritional demands during this critical period.

Keywords: protected amino acids, 3-methylhistidine, muscle mobilization, late pregnancy

Introduction

Dairy cows in the late stages of gestation often exhibit weight gain due to the logarithmic growth of the gravid uterus. How can we determine if a cow has experienced weight loss due to nutrient allocation to her fetus? There is a particular need for accurate definition of the pattern of fetal and total conceptus growth during late pregnancy in Holstein cows.

In late gestation, the higher rate of growth in the gravid uterus is characterized by increased nutrient requirements and decreased dry matter intake (DMI). This, combined with hormonally regulated and genetically driven metabolic adaptations, leads to the mobilization of body nutrient reserves (Drackley, 1999; Hayirli et al., 2002). The factor that initiates the breakdown of existing proteins within muscle tissue and other protein reserves is primarily the inadequacy in amino acids (AA), especially essential AA, in the bloodstream. This process is regulated by various metabolic pathways and signaling mechanisms, including the mammalian target of rapamycin (mTOR) pathway (Tee, 2018), which acts as a central regulator of protein synthesis and degradation in response to nutrient availability, particularly AA. Thus, the absence or depletion of essential AAs in the bloodstream, when dietary crude protein (CP) intake is insufficient, serves as the primary signal for initiating the breakdown of existing proteins in tissues such as muscles. Megahed et al. (2019) proposed that mobilization of intramuscular fatty acids (FA), as estimated by changes in muscle echogenicity, could contribute to muscle depth loss. Excessive mobilization of fats, proteins, glycogen, and minerals is often associated with immune dysfunction, increasing the cows' susceptibility to both metabolic and infectious diseases postpartum (Bradford and Swartz, 2020). The degree of this contribution in dairy cows, if any, has to be investigated. Siachos et al. (2024) reported that increased 3-MH at 7 days prior to parturition was associated with greater risk for metritis and at least one clinical disease during the first 28 d post-calving; this time point coincided with the initiation of skeletal muscle mobilization. Muscle tissue accounts for approximately 60% of the total metabolically- active body mass. Consequently, despite the relatively low basal metabolic rate, any alterations in muscle composition or size can impact the overall body metabolism (Davis and Fiorotto, 2005).

Despite the growing body of research in this field, the variation of 3-MH in transition dairy cows has not been adequately described. Previous studies relied on single sampling points and have not examined how the weeks leading up to parturition affect the 3-MH concentration. Detailed biochemistry of protein metabolism during the transition period is lacking (Siachos et al., 2024). Therefore, this study aimed to: (1) investigate changes in biomarkers indicative of protein metabolism (3-MH, total protein, albumin, PUN, and creatinine) during the last month of pregnancy in Holstein cows subjected to varying protein feeding diets; (2) evaluate the association between each biomarker and skeletal muscle reserves, as estimated by blood metabolite measurements; and (3) assess the relationship between these biomarkers and changes in body weight (BW) pre-partum.

Materials and methods

Management and experimental design

The experiment was conducted in the countryside of Isfahan, Iran, at an elevation of 1,608 meters above sea level, with coordinates of 32° 65′ N latitude and 51° 45′ E longitude. Multiparous, late-pregnant, dry Holstein cows (781 kg ± 16.22 kg BW and 3.46 ± 0.18 BCS) were allotted to three rations, 1) a standard diet as the control ration (Cont.), 2) a diet containing rumen-protected AAs (methionine and lysine; PAA), formulated based on the latest recommendations to provide 1.14 g of methionine and 3.03 g of lysine per megacalorie of metabolizable energy (ME), regardless of the levels provided by feed ingredients in the control diet, and 3) a high-crude protein (High-CP) diet, using plant source protein such as soybean meal, rapeseed meal, and corn gluten meal, which supplied double the CP level of the control diet. Therefore, the CP level in the diet was inevitably increased to about 27%. The rations, formulated by using the CPM Dairy® software, were fed as a total mixed ration (TMR) at ad-libitum, and provided in three meals per day. The cows had free access to fresh water throughout the experiment. The Guide for the Care and Use of Agricultural Animals in Research and Teaching (FASS, 2010) was followed for all aspects of this study.
Each treatment contained three cows in their third parity and four cows in their fourth parity. To ensure consistency, the cows assigned to the treatments had similar average production levels in the previous 305 days of lactation, similar BCS, and no reproductive issues in previous calving. The cows were housed in individual stalls from 28 days before parturition until calving. Sample collection started on day 28 before parturition, and the traits were measured weekly during the four-week study period.

The experimental diets for this period are detailed in Table 1. The lysine and methionine profiles of the feed items were determined using the near infra-red (NIR) method.

Immediately after calving, the cows underwent thorough examination and documentation, including placental discharge and weight, calf BW, colostrum production and consumption by the calf, and calving ease. Feed was continuously provided to ensure the mangers were never empty, with less than 5% of feed remaining within 24-hour period.

Sampling and measurements

Each animal was weighed weekly, from four weeks before calving, at early morning and before feeding [electronic scale designed for large animals, offering precision up to ±200 grams (Damkesh Eatemad®)]. The BCS was recorded on a 5-point scale, with a score of 1 indicating very thin cows and a score of 5 for very fat cows (Edmonson et al., 1989). Fat depth (in the sacral region proximal to the first coccygeal vertebra) and the eye muscle (*Longissimus dorsi*) depth (between ribs 12 and 13), was measured ultrasonographically (Easi-Scan® BSF) on the same day the cows were weighed (Van der drift et al., 2012; Megahed et al., 2019). The DMI and ort were measured daily and sampling was done weekly. The feed samples (or orts) were mixed thoroughly for chemical analysis. Dry matter content was determined by drying in an oven equipped with a fan at a temperature of 68°C for 48 hours. Then it was ground by a 1 mm mill (Wiley, Pulverizer for laboratory, Orgaw Seiki Co., Ltd., Tokyo, Japan). The ash content was determined by burning the sample in an electric furnace at 550 °C. Crude protein and ether extract contents were determined by the Kjeldahl and Soxhlet methods, respectively (AOAC, 1990). Neutral detergent insoluble fiber (NDF) and acid detergent insoluble fiber (ADF) were also measured (Van Soest et al., 1991).

Table 1. Ingredients and chemical composition of the total mixed rations fed to late- pregnant cows

	Cont.	PAA	High-CP
Feedstuff (g/kg DM basis)			
Alfalfa hay	393.7	391.3	204.4
Corn silage	289.1	287.4	150.1
Wheat straw	73.8	73.3	38.3
Beet pulp	3.5	3.4	1.8
Rice bran	70.4	69.9	36.5
Cotton seed	3.6	3.6	1.9
Corn grain	21.7	21.6	11.3
Corn gluten meal	7.2	7.2	79
Soybean meal	14.1	14	145.9
Rapeseed meal	88.4	87.9	296.1
Protected methionine	.	2.9	.
Protected lysine	.	8.4	.
Calcium carbonate	6.8	6.8	17.8
Calcium chloride	6.3	3.8	4.6
Magnesium oxide	1.1	1.1	0.6
Magnesium sulfate	12.2	9.4	7.5
Sodium bentonite	1.1	1.1	0.6
Monensin	0.1	0.1	.
Vitamin mixture	3.45	3.4	1.8
Mineral mixture	3.45	3.4	1.8
Chemical and nutritional composition (g/kg DM)			
Dry matter[1]	519.7	521.2	659.5
Crude protein[1]	133.2	146	272
RDP[2](g/kg CP)	738.4	711.3	620.5
RUP[2](g/kg CP)	261.6	288.7	379.5
Lysine (g/kg MP)	50.5	77.7	37.8
Methionine (g/kg MP)	16.7	30.8	14
Neutral detergent fiber[1]	496.6	493.6	377
Acid detergent fiber[1]	284.3	279.4	168.5
Non-fiber carbohydrates [2]	236	234	255.3
Starch[2]	93.2	92.5	100.2
Ash[1]	96.5	90.6	63.5
DCAD[2] (meq/kg DM)	-100.4	-101.0	-100.5
Metabolizable energy[2] (MJ/kg)	7.91	8.03	8.91

Cont.: Control; PAA: protected amino acid; High-CP: high dietary crude protein; RDP: rumen degradable protein; RUP: rumen undegradable protein; MP= metabolizable protein; DCAD= dietary cation- anion difference.

[1]Laboratory measurement

[2]Calculated from NRC tables (NRC, 2021)

To determine the blood metabolites, blood was taken weekly, approximately 3.5 hours after the morning feeding, from the abdominal vein in Venoject® tubes containing heparin. The tubes were centrifuged at 2000 rpm for 15 minutes and plasma kept at -20°C.

Plasma samples were analyzed for 3-MH, albumin, beta hydroxybutyrate (βHB), urea nitrogen, cholesterol, creatinine, glucose, NEFA, total proteins, triglyceride and uric acid. Beta-hydroxybutyrate was measured with Ranbut commercial kit (Randox®, England) and high performance liquid chromatography (HPLC), was used for 3-MH measurement (Houweling et al., 2013). Glucose, urea, creatinine, bilirubin, uric acid, albumin and total protein were measured with commercial kits (Pars Azmoun®, Tehran, Iran).

Urine samples were collected at designated intervals for total nitrogen, creatinine, uric acid, urea, and allantoin measurement (Valadares et al., 1999; Chizzotti, 2008; Lee et al., 2019). At parturition, colostrum was immediately milked and weighed, while the weights of the placenta, calf, and the calving fluids, including both amniotic and allantoic components, were measured. Calving fluid was collected using a large pan positioned on the ventral aspect of the cow's body. Colostral freezing point was measured, and samples were analyzed for fat, protein, lactose and 3-MH amounts (Foss Electric, Denmark).

The metabolic fecal nitrogen (MFN) was estimated by analyzing the nitrogen content in the feces and regressing the intake of digestible nitrogen on the intake of CP with the regression intercept representing the estimated MFN (NRC, 2001). The endogenous urinary nitrogen (EUN) was estimated by analyzing the nitrogen content in the urine and regressing the intake of nitrogen on the intake of CP. The intercept represented the estimated EUN. In this manner, a factorial approach was used to estimate nitrogen requirements for pregnant cows. The nitrogen requirements for each cow are based on the maintenance requirements (MFN + EUN + Scurf N) plus requirements of the gravid uterus (NASEM, 2021). Nitrogen retention in the body was calculated as the difference between nitrogen intake and the total nitrogen excreted through urine, feces, and scurf.

Statistical analysis

The study was conducted with seven replications over a period of four weeks. For certain parameters, a factorial arrangement (two parties and three rations) in a completely randomized design was employed. Data were analyzed using the Proc MIXED in SAS (version 9.4; SAS Institute Inc., Cary, NC) with repeated measures. The statistical model included the fixed effects of the treatments, time, and their interactions, and cow was considered as the random effect. Non-repeated measurements across the weeks were analyzed using the Proc GLM. Mean comparisons were performed using the least squares means procedure adjusted for the Tukey's test. The level of significance was set at P<0.05, and a tendency was noted at 0.05<P<0.15. Additionally, correlations between certain parameters were assessed using the proc CORR, with correlation coefficients tested using the Student's t-test.

Results

The DMI progressively decreased during last gestation, with a notable reduction of about 25% around calving compared to three weeks earlier. However, no significant differences in BW or BCS were observed between the starting point and calving times (Table 2). Notably, the back fat depth remained unchanged during this period, although a significant reduction was observed in the last week of pregnancy (P<0.01). Cows on a High-CP diet exhibited the highest values for the both back fat and eye muscle depths, showing significant differences compared to other treatments (P<0.01). There was no significant interaction between the time period and treatments (P>0.05).

When shifting focus from the external appearance to the internal metabolic profile of the cows, a different perspective emerged in their blood (Table 3). Blood metabolites associated with anabolic processes, such as albumin, PUN, cholesterol, triglyceride, and total protein, exhibited progressive reductions in the weeks leading up to calving. Conversely, metabolites linked to catabolism, such as creatinine, βHB, and NEFA, showed increasing trends during this period. Glucose and uric acid concentrations remained stable, indicating minimal impact on these metabolites. The 3-MH concentration increased steadily week by week until parturition (P<0.001). While there was no significant interaction between the time period and treatments, treatments significantly affected βHB, PUN, cholesterol, and triglyceride levels (P<0.05). Cows on a High-CP diet exhibited the highest PUN concentration, as expected, compared to other treatments. No significant differences were observed in other protein metabolism-related metabolites such as 3-MH, albumin, creatinine, and uric acid, except for PUN.

Table 2. The effects of treatments and days relative to calving on BW and BCS changes, feed intake, and back- fat and eye muscle depth in Holstein cows

Item	Days relative to parturition (Period)						Treatments					P×T
	-21	-14	-7	0	SEM	*P*-Value	Cont.	PAA	High-CP	SEM	*P*-Value	
Body weight (kg)	781 ^{c}	793 ^{b}	807 ^{a}	787 ^{cb}	3.006	<.0001	789 ^{b}	787 ^{b}	801^{a}	2.603	0.0070	0.6622
Body condition score	3.46 ^{b}	3.46 ^{b}	3.62 ^{a}	3.48 ^{ab}	0.041	0.0202	3.47	3.54	3.50	0.035	0.3526	0.4321
Dry matter intake (kg/d)	17.01 ^{a}	15.68 ^{b}	14.03^{c}	12.96^{d}	0.271	<.0001	13.81 ^{b}	14.55 ^{b}	16.41^{a}	0.234	<.0001	0.7343
Back- fat depth (mm)	46.48	26.97	27.66	26.57	0.645	0.5586	26.64 ^{b}	25.00 ^{b}	29.11^{a}	0.559	<.0001	1.0000
Eye muscle depth (mm)	46.34 ^{a}	45.71 ^{a}	45.24^{a}	43.20 ^{b}	0.469	<.0001	45.39 ^{a}	43.70 ^{b}	46.28^{a}	0.406	<.0001	0.9946

Cont.: Control; PAA: protected amino acid; High-CP: high dietary crude protein; SEM: standard error of the mean; P×T: interaction between period and treatment.
a,b: Within rows in each category, means with common superscript(s) are not different (P>0.05, Tukey's test).

Table 3. The effects of treatments and days relative to calving on the concentration of several plasma metabolites in Holstein cows

Item	Days relative to parturition (Period)						Treatments					P×T
	-21	-14	-7	0	SEM	*P*-value	Cont.	PAA	High-CP	SEM	*P*-value	
Albumin (g/L)	36.80^{a}	35.99 ^{a}	35.16 ^{a}	31.59 ^{b}	0.513	<.0001	34.01	35.71	34.94	0.584	0.1486	0.8185
β- Hydroxy butyrate (mmol/L)	0.584 ^{b}	0.691 ^{ab}	0.821 ^{a}	0.972 ^{a}	0.373	<.0001	0.850^{a}	0.626^{b}	0.723^{b}	0.034	0.0008	0.6684
Cholesterol (mg/dL)	70.67 ^{a}	61.27 ^{b}	54.34 ^{c}	52.36 ^{c}	1.054	<.0001	62.47^{a}	58.29^{b}	58.23^{b}	1.187	0.0342	0.1993
Creatinine (mg/ dL)	64.44 ^{b}	64.42 ^{b}	69.13 ^{a}	69.33 ^{a}	0.670	<.0001	67.74	65.53	67.23	1.151	0.3853	0.0643
Glucose (mg/ dL)	66.55 ^{b}	66.92 ^{b}	67.45 ^{b}	78.21 ^{a}	0.948	<.0001	69.27	70.04	70.20	1.375	0.8799	0.1127
NEFA (mmol/L)	0.110 ^{c}	0.125 ^{c}	0.190 ^{b}	0.293 ^{a}	0.017	<.0001	0.207	0.162	0.169	0.015	0.1135	0.4089
PUN (mg/ dL)	15.44^{a}	14.93 ^{b}	14.21 ^{b}	12.63^{c}	0.289	<.0001	13.14^{b}	13.89^{b}	15.87^{a}	0.388	0.0003	0.2901
Triglycerides (mg/ dL)	42.20 ^{a}	44.15 ^{a}	42.53 ^{a}	39.14 ^{b}	0.690	<.0001	40.10^{b}	44.89^{a}	41.03^{b}	0.803	0.0012	0.0236
Total protein (g/L)	78.10 ^{a}	75.53 ^{b}	74.82^{bc}	73.08 ^{c}	0.838	<.0001	73.57	76.79	75.79	1.214	0.1879	0.7656
3-MH (µmol/L)	4.631^{d}	6.102 ^{c}	7.153 ^{b}	10.15 ^{a}	0.368	<.0001	7.475	6.400	7.152	0.528	0.3580	0.9998
Uric acid (mmol/L)	1.431	1.422	1.342	1.397	0.033	0.1904	1.328	1.429	1.437	0.035	0.0734	0.3958

Cont.: Control; PAA: protected amino acid; High-CP: high dietary crude protein; SEM: standard error of the mean; PUN: plasma urea nitrogen; 3-MH: 3-methylhistidine; P×T: interaction between period and treatment.
a,b: Within rows in each category, means with common superscript(s) are not different (P>0.05, Tukey's test).

The data on urine metabolites (Table 4), showed excretion of the typical end products of metabolism filtered by the kidneys. Except for creatinine, the urinary concentrations of allantoin, total urea nitrogen, and uric acid decreased significantly (P<0.001) in all treatments from the beginning of the experiment until calving time. Although the interaction between treatments and periods was not significant (P>0.05), all urinary metabolites indicated significant effects of the treatments (P<0.05). Cows on the High-CP diet exhibited the highest concentrations of urea nitrogen and total nitrogen in their urine, as expected, compared to other treatments (P<0.001).

Table 4. The effects of treatments and days relative to calving on several urinary metabolites in Holstein cows

	Days relative to parturition (Period)						Treatments					
Item	-21	-14	-7	0	SEM	*P*-value	Cont.	PAA	High-CP	SEM	*P*-value	P×T
Allantoin (mmol/L)	14.69[a]	13.72[b]	12.54[c]	11.69	0.236	<.0001	12.20[c]	13.23[b]	14.05[a]	0.212	<.0001	0.9807
Creatinine (mg/dL)	89.00[b]	90.02[b]	95.54[a]	94.46[a]	0.800	<.0001	92.92[ab]	90.38[b]	93.47[a]	0.717	0.0008	0.6086
Total nitrogen (g/L)	9.66[a]	9.06[b]	8.54[b]	7.92[c]	0.148	<.0001	8.15[b]	8.51[b]	9.73[a]	0.138	<.0001	0.0190
Urea (g/l)	6.56[a]	5.95[b]	5.28[c]	4.92[c]	0.109	<.0001	4.16[c]	5.30[b]	7.57[a]	0.976	<.0001	0.1333
Uric acid (mmol/L)	2.59[a]	2.18[b]	2.21[b]	2.07[b]	0.040	<.0001	2.16[b]	2.33[a]	2.30[a]	0.036	0.0734	<.0001

Cont.: Control; PAA: protected amino acid; High-CP: high dietary crude protein; SEM: standard error of the mean; P×T: interaction between period and treatment.
a,b: Within rows in each category, means with common superscript(s) are not different (P>0.05, Tukey's test).

The nitrogen status was investigated as a crucial factor during the transition period (Table 5). Initially, nitrogen intake (CP) was higher due to increased feed consumption. With advance pregnancy, feed consumption decreased, resulting in a corresponding decrease in nitrogen intake, with a significant difference between periods. Protein consumption during the last week of pregnancy decreased by approximately 25%. The EUN showed minimal fluctuation in the weeks leading to calving, while MFN remained consistent until the final week of pregnancy, where it peaked. Fecal nitrogen excretion initially peaked at the start of the experiment, decreased after two weeks, and remained constant until calving. Protein digestibility remained consistent throughout the drying period, showing no significant differences between periods.

The maintenance nitrogen requirement EUN, scurf nitrogen (from skin, skin secretions, and hair), and MFN, significantly increased in the last week of gestation, coinciding with a dramatic decrease in nitrogen intake compared to the initial two weeks of the experiment (P<0.05). Urinary nitrogen excretion gradually decreased as calving approached. Nitrogen retained in the gravid uterus increased steadily week by week during pregnancy, reaching its peak in the final week (P<0.05). Simultaneously, nitrogen retained in the body showed a gradual decrease, reaching its lowest value in the last week of pregnancy (P<0.01).

With increased uterine weight and nitrogen deposition the cows lost BW and nitrogen. Cows receiving PAA treatment exhibited the least amount of nitrogen loss from their bodies, while those on the control ration showed the highest nitrogen loss.

Table 5. The effects of treatments and days relative to calving on the nitrogen status in Holstein cows

	Days relative to parturition (Period)						Treatments					
Item	-21	-14	-7	0	SEM	*P*-value	Cont.	PAA	High-CP	SEM	*P*-value	P×T
Endogenous urinary nitrogen (g/d)	41.11	40.99	42.31	41.99	0.485	0.1555	41.16	41.31	42.32	0.420	0.1103	0.9183
Metabolic fecal nitrogen (g/d)	17.94[b]	18.09[b]	18.85[b]	21.77[a]	0.671	0.0003	20.41[a]	17.92[b]	19.15[ab]	0.581	0.0130	0.3469
Fecal nitrogen (g/d)	254[a]	241[a]	198[b]	204[b]	10.80	0.0006	99[b]	106[b]	467[a]	9.356	<.0001	0.0431
Crude protein digestibility (%)	56.52	55.50	58.62	55.01	2.288	0.6910	67.02[a]	68.24[a]	33.99[b]	1.981	<.0001	0.9459
Nitrogen for maintenance (g/d)	60.53[b]	60.56[b]	62.66[ab]	65.24[a]	0.811	0.0002	63.06[a]	60.71[b]	62.98[a]	0.702	0.0324	0.5602
Total urine nitrogen (g/d)	254[a]	231[b]	222[b]	189[c]	6.413	<.0001	200[b]	229[a]	242[a]	5.553	<.0001	0.6940
Nitrogen intake (g/d)	504[a]	467[b]	415[c]	388[c]	8.485	<.0001	294[c]	322[b]	705[a]	7.348	<.0001	0.0218
Nitrogen retained in body (g/d)	-5.61[a]	-5.66[a]	-6.16[ab]	-6.40[b]	0.156	0.0009	-6.27[b]	-5.68[a]	-5.93[ab]	0.135	0.0107	0.3474
Nitrogen retained in gravid uterus (g/d)	19.84[d]	21.66[c]	23.94[b]	26.44[a]	0.287	<.0001	21.59[c]	24.26[a]	23.07[b]	0.249	<.0001	0.9947

Cont.: Control; PAA: protected amino acid; High-CP: high dietary crude protein; SEM: standard error of the mean; P×T: interaction between period and treatment.
a,b: Within rows in each category, means with common superscript(s) are not different (P>0.05, Tukey's test).

Table 6 presents data on the cow demographics, including the weight of newborn calves and their initial colostrum intake. The calf BW was influenced by the treatment, with the highest weight recorded in calves from PAA cows (P<0.01). No significant difference was observed in calf BW between the control; and High-CP cows. Colostrum consumption was not affected by the treatments.

Significant differences were observed in colostral composition, specifically in protein percentage, 3-MH concentration, and freezing point. Cows consuming PAA had the highest percentage of protein in their colostrum, which did not differ from the High-CP diet; however, both treatment groups differed significantly from the control group (P<0.01). A significant difference in colostral protein percentage was noted between the third and fourth parity cows, with cows in the fourth parity showing higher protein percentages. Cows on PAA diets had the lowest and the control group recorded the highest colostral freezing point (P<0.01). The concentration of 3-MH was the lowest in cows consuming PAA ration, intermediate in High-CP diets cows, and the highest in the control group (P<0.01). No significant differences were found in 3-MH concentration with respect to parity; there were also no significant interactions between treatments and parity.

Table 6. The effects of dietary treatment and cow parity on calf weight, and colostrum intake and composition in Holstein cows

	Treatments					Parity				
Item	Cont.	PAA	High-CP	SEM	*P*-value	Third	Forth	SEM	*P*-value	T×P
Calves body weight (kg)	40.89 [b]	45.96 [a]	43.70 [a b]	0.984	0.0060	43.593	43.457	0.802	0.9058	0.4531
Colostrum intake (kg)	4.286	4.643	4.643	0.125	0.1037	4.556	4.500	0.102	0.7073	0.9909
Colostral Composition										
Protein (%)	15.10 [b]	17.95 [a]	16.73 [a]	0.536	0.0057	15.83 [b]	17.16 [a]	0.437	0.0490	0.6131
Fat (%)	6.32	6.21	5.93	0.324	0.7572	6.01	6.26	0.264	0.5259	0.6014
Lactose (%)	2.61	2.73	2.69	0.207	0.9569	2.68	2.67	0.169	0.9392	0.7390
Freezing point (°C)	-0.560 [a]	-0.589 [b]	-0.572 [a b]	0.006	0.0144	-0.569	-0.577	0.005	0.2413	0.1703
3-MH (µmol/L)	1.904 [a]	0.803 [c]	1.253 [b]	0.068	<.0001	1.387	1.270	0.056	0.1602	0.5223

Cont.: Control; PAA: protected amino acid; High-CP: high dietary crude protein; 3-MH: 3-methylhistidine; SEM: standard error of the mean; P×T: interaction between parity and treatment.
a,b: Within rows in each category, means with common superscript(s) are not different (P>0.05, Tukey's test).

Strong significant positive correlations (r=0.67, P<0.0001) were found (Figure 1) between nitrogen retained in the gravid uterus (g/d) and plasma concentration of 3-MH (µmol/L). The most suitable mathematical model for describing this relationship is characterized by a modified power function:

$$\text{Plasma 3-MH } (\mu mol/L) = 1.163 \times (1.081)^x$$

where, x represents nitrogen retained in the gravid uterus in grams per day.

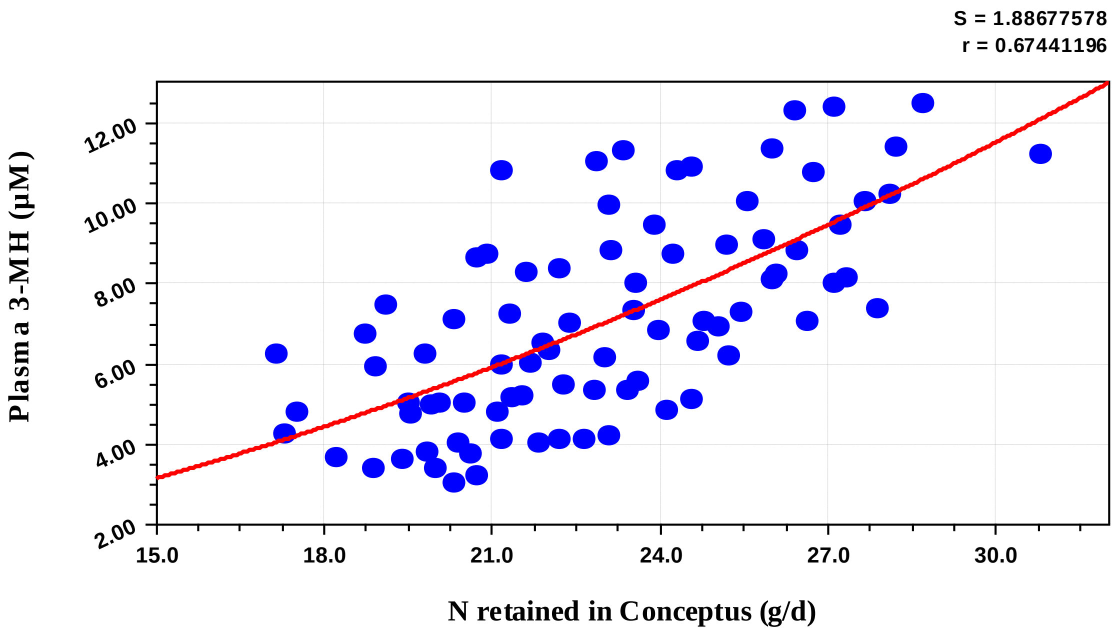

Figure 1: Relationship between nitrogen retained in the conceptus and plasma concentration of 3-methylhistidine (3-MH).

Discussion

It has been nearly three decades since Bell et al. (1995) reported that the NRC (1988) and other existing feeding systems for dairy cows assume constant feed intake and nutrient requirements throughout the non-lactating period, with body protein reserves considered static during the dry period. Contrary to the older assumption of static reserves, it is now understood that body protein reserves can fluctuate in response to varying metabolic demands, such as the growth of the gravid uterus, and nutritional management (Sadri et al., 2023). This dynamic nature of body protein reserves highlights the need for more adaptive feeding strategies to meet the changing nutrient requirements of pregnant cows during the dry period.

As shown in Table 2, the weight of the cows remained almost constant during the 4-week drying period. However, considering the growth of the gravid uterus, which is approximately 70 kilograms (including the mean calf weight of 43.25 kg, amniotic fluid of 17.65 kg, and placenta of 6.55 kg), it is evident that the actual BW of the cows decreased during this period. This BW loss was effectively transferred to the gravid uterus. The present data are consistent with Pires et al. (2013), who reported similar fluctuations in BCS and BW of cows in their study. Despite the stable BCS throughout this period, it can be concluded that the BCS is not a highly reliable indicator for assessing the cows' condition during the dry period due to the significant growth of the gravid uterus.

No significant changes were observed in the back fat depth during the dry period. In the final two weeks of pregnancy, all cows exhibited an increase in the concentrations of βHB and NEFA, while triglyceride and cholesterol levels decreased (Table 3). This suggests heightened energy demands, particularly from the gravid uterus, necessitating cows to mobilize body reserves due to reduced DMI. Fat mobilization in cows is increased by having a higher BCS before calving (Komaragiri et al., 1998; Kokkonen et al., 2005). Despite these demands, back fat is not prioritized as the primary reserve for meeting the energy requirements. Cows fed a High-CP diet had a higher back fat depth than the other groups, indicating that some of the nutrient intake was converted to fat and stored in this area. These finding is in agreement with Drackley (2011) who observed that even modest energy excess during the dry period can lead to significant internal fat deposition without detectable changes in body condition. Drackley (2011) concluded that it is essential to meet the requirements for energy and other nutrients during the dry period. There was no significant change in eye muscle depth from the start of the experiment until one week before calving, but a significant reduction was observed in the week before parturition. It should be noted that cows fed a High-CP diet had higher BW and DMI than the other groups, demonstrating the impact of protein in their diet; however, it remains to be determined whether this level of protein consumption is advisable. However, earlier studies showed that the composition of prepartum or early lactation diets could influence fat mobilization (Komaragiri et al., 1998; Van Knegsel et al., 2007).

The amino acid 3-MH is a product of actin and myosin degradation that is not reused for protein synthesis. Urinary excretion of 3-MH around calving is an indicator of protein mobilization in early lactation (Chibisa et al., 2008). Total urine collection is not feasible in most trials involving periparturient cows; therefore, plasma 3-MH concentration has been used as an indicator of muscle mobilization in cows (Blum et al., 1985; van der Drift et al., 2012; Pires et al., 2013; Sadri et al., 2023; Siachos et al., 2024). As shown in Table 3, 3-MH levels increased weekly until calving, while total protein levels in plasma declined. Notably, plasma concentration of total protein did not exhibit a difference between weeks 3 and 4 of the experiment, highlighting 3-MH as a superior indicator for assessing muscle mobilization. In this context, plasma albumin levels were also found to be inconsistent indicators of muscle mobilization, with significant decreases occurring only one week before calving, when protein demand was particularly high. Albumin is a negative acute phase protein, meaning its liver synthesis is downregulated during infection or inflammation, whereas globulin consists of some of the main positive acute phase proteins (Ceciliani et al., 2018). Albumin has a half-life of 16.5 d in bovine plasma (Cornelius et al., 1962); therefore, any alterations in liver synthesis rate require several days to manifest. Notably, despite the high CP intake in both the High-CP and PAA groups, significant changes in muscle mobilization persisted, with no substantial differences observed between the treatments ($P>0.05$). Therefore, recent findings suggest that protein metabolism in transition cows is more intricate than previously thought, varying both between individual cows and depending on the specific ration applied.

In this context, Figure 1 clearly shows that as the amount of nitrogen retained in the gravid uterus increased, the level of 3-MH in the blood plasma also increased, indicating a significant positive correlation. The coefficient of determination (r^2) is the best measure to explain the degree of association between two variables (Moharrery and Das, 2002). For 3-MH and nitrogen retained in the gravid uterus, the calculated r^2 is 0.454. This means that 45.4% of the total variation in 3-MH concentration can be explained by the protein demand for the growing gravid uterus. The remaining of the variation in 3-MH concentration may be related to other aspects of protein turnover in the body. This includes overall protein turnover rates, muscle protein catabolism, and basal protein metabolism. Variability in these processes, which encompasses differences in protein synthesis and degradation, as well as individual metabolic rates and dietary protein utilization, likely contributes to the unexplained portion of the variation in 3-MH levels. In the current experiment, plasma 3-MH presented the typical profile of the periparturient period and concentrations were within the ranges found by others (Bell and Bauman, 1997; Doepel et al., 2002, Pires et al., 2013).

The high demand for CP and AAs in pregnant cows was reflected in the decreased excretion of nitrogenous compounds in their urine (Table 4). All nitrogenous compounds showed a declining trend week by week until calving, except for creatinine concentration, which indicates tissue catabolism and reserve tissue mobilization. In alignment with the present findings, Edouard et al. (2020) reported that dairy cows can tolerate significant deficits in protein supply in maize-based diets for up to two weeks without a substantial decline in performance, as long as metabolizable protein levels are maintained at threshold levels. During periods of high protein demand, cows may have utilized urea recycling for microbial protein synthesis, thereby compensating for the protein deficit in their bodies and reducing urine nitrogen excretion. In this manner, significant differences were observed in urinary uric acid excretion among dietary treatments. Uric acid, a purine derivative, serves as an indicator of microbial protein synthesis in the rumen (Topps and Elliott, 1965). The observed changes in uric acid excretion suggest an increase in the supply of rumen-degradable nitrogen, which was partially utilized by rumen microbes. However, the majority of the degraded nitrogen was absorbed as ammonia and subsequently excreted as urea in the urine. Despite elevated nitrogenous compounds in the blood, all cows exhibited signs of body mobilization, reflected in 3-MH or creatinine concentrations. Cows fed the control diet did not differ significantly from those on the High-CP ration. However, cows receiving PAA exhibited improved conditions regarding urine nitrogen excretion compared to the other treatments under the experimental conditions.

The cows on High-CP diet, which contained 27% CP (twice the typical amount for dry cows' rations), showed greater nitrogenous loss in the feces. This suggests that, despite the high protein intake from plant sources such as soybean meal, rapeseed meal, and corn gluten meal (which are less digestible) increased protein supply did not significantly improve the absorbable protein available to the cow, and their ability to digest protein may be limited (Table 5). The lower digestibility of these plant-based proteins led to inefficient protein utilization and higher nitrogen excretion. Feeding a high CP diet based on soybean meal can maintain the nutritional requirements of dairy cows; however, while in a state of negative AA balance, the efficiency of protein utilization is low (Titgemeyer et al., 1989). This underscores the importance of incorporating more easily digestible protein sources into the diet to optimize protein absorption and reduce its wasting. An interesting finding from the study is that the level of EUN remained unchanged throughout the entire pregnancy. However, a significant difference in MFN levels was observed in the week leading to calving compared to previous periods, indicating an increased need for maintenance nitrogen during this crucial period. A part of urinary excretion (20-30%) is of EUN, but the main variations of total urinary nitrogen excretion are linked to those of digestive and metabolic processes (Faverdin and Verite, 1998), and corresponds to urea-N. The sharp decrease in urinary nitrogen excretion in the last week of pregnancy can be attributed to two factors: reduced DMI and consequently lower nitrogen intake, leading to decreased excretion through urine, as well as the increased internal demand for nitrogenous compounds. Typically, voluntary feed intake in *Bos taurus* females decreases with approaching parturition (Stanley et al., 1993), possibly due to limited ruminal space caused by the gravid uterus expansion. In contrast, there is evidence that physical constraints on feed intake during late gestation may be compensated by an increased passage rate (Linder et al., 2014). In the tropics, studies have revealed that protein supplementation may improve forage intake by improving the adequacy of substrates (i.e., energy and protein) in both metabolism and the rumen (Detmann et al., 2014).

In the final week of pregnancy, there is also a notable increase in nitrogen storage in the gravid uterus. There is a negative trend in nitrogen balance, indicating more nitrogen retention due to the rapid growth of the gravid uterus, despite the protein content in the ration potentially being insufficient to support such exponential growth. This shortfall leads to mobilization of muscle tissue to supply the necessary AAs. However, supplementation of the diet with PAA significantly enhances nitrogen storage intensity in the gravid uterus. Lysine, as the main limiting AA in ruminants (NRC, 2001), is an important precursor for protein synthesis in dairy cows (Lin et al., 2018). Rumen-protected lysine supplementation could increase the duodenal lysine flow and improve the extraction efficiency of other essential AAs by other body tissues (Guinard and Rulquin, 1994).

Experimental data indicated that a significant portion of the protein intake in cows on a high-CP diet is excreted through feces (undigested), with only a small portion excreted through urine, albeit this urinary nitrogen excretion remains significant compared to the control group.

The effects of treatments were also evident post-calving in cows, reflected in the increased weight of calves fed rations containing High-CP or PAA, showing significantly higher weights (Table 6). Additionally, their colostrum exhibited a higher protein content. Conversely, cows in the control group had the highest concentration of colostral 3-MH, followed by those receiving the High-CP rations, while the lowest 3-MH concentration was observed in the cows supplemented with PAA. This suggests that 3-MH can enter milk via the bloodstream. However, higher protein concentrations in colostrum are associated with a reduced freezing point. Colostrum production, or colostrogenesis, begins approximately three to four weeks before calving and ceases gradually after parturition (Brandon et al., 1971). In multiparous cows, this process occurs during the dry period. Current recommendations from NASEM (2021) on prepartum protein supply for late-pregnant cows do not account for the protein requirements specific to colostrum production, as there is insufficient data on the impact of dietary protein on colostrum yield. Previous studies showed minimal effects of prepartum dietary protein supplementation on early lactation milk and protein yield (Bell et al., 2000; Lean et al., 2013). However, prepartum protein supplementation has been shown to improve the health status of the fresh cows (Lean et al., 2013). While previous research has concentrated on the effects of prepartum protein supplementation on subsequent lactation performance (Huyler et al., 1999; Santos et al., 2001; Doepel et al., 2002), there has been less focus on its impact on colostrum production and calf performance. Additionally, inadequate metabolizable protein supply negatively affects the immune system and is associated with an increased risk of peripartal diseases (Drackley and Cardoso, 2014).

Conclusion

Based on the detailed findings, this study revealed dynamic metabolic shifts in dairy cows during the last month of pregnancy. Despite stable body metrics such as BW and back fat thickness, the cows exhibited increased plasma 3-MH levels and other parameters related to protein mobilization, indicating significant muscle and fat mobilization leading to weight loss. Urinary nitrogen excretion decreased, suggesting altered protein metabolism, while colostrum from cows on High-CP or PAA diets showed higher protein content. These findings highlight that during the final month of pregnancy, increasing the protein level in dairy cows' rations up to twice the standard recommendation, or supplementing with rumen-protected AAs at twice the standard dose, does not prevent muscle mobilization. However, such dietary adjustments can mitigate the severity of muscle mobilization during this critical period.

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References

AOAC., 1990. Official Methods of Analysis. Vol. I. 15th ed. Association of Official Analytical Chemists. Arlington, VA, USA.

Bell, A.W., Bauman, D.E., 1997. Adaptations of glucose metabolism during pregnancy and lactation. *Journal of Mammary Gland Biology, Neoplasia* 2, 265-278.

Bell, A.W., Burhans, W.S., Overton T.R., 2000. Protein nutrition in late pregnancy, maternal protein reserves and lactation performance in dairy cows. *Proceedings of the Nutrition Society* 59,119-126. http:// doi.org/10.1017/ S0029665100000148.

Bell, A.W., Slepetis, R., Ehrhardt, R.A., 1995. Growth and accretion of energy and protein in the gravid uterus during late pregnancy in Holstein cows. *Journal of Dairy Science* 78, 1954-1961.

Blum, J.W., Reding, T., Jans, F., Wanner, M., Zemp, M., Bachmann, K.,1985. Variations of 3-methylhistidine in blood of dairy cows. *Journal of Dairy Science* 68, 2580-2587. https://doi.org/10.3168/jds.S0022-0302(85)81140-1.

Bradford, B.J., Swartz, T.H., 2020. Review: Following the smoke signals: Inflammatory signaling in metabolic homeostasis and homeorhesis in dairy cattle. *Animal* 14(S1), s144-s154. https://doi.org/10.1017/ S1751731119003203.

Brandon, M.R., Watson, D.L. Lascelles, A.K., 1971. The mechanism of transfer of immunoglobulin into mammary secretion of cows. *Australian Journal of Experimental Biology and Medical Science* 49, 613-623. https://doi.org/10.1038/icb.1971.67.

Ceciliani, F., Lecchi, C., Urh, C., Sauerwein, H., 2018. Proteomics and metabolomics characterizing the pathophysiology of adaptive reactions to the metabolic challenges during the transition from late pregnancy to early lactation in dairy cows. *Journal of Proteomics* 178, 92-106. https://doi.org/10.1016/j.jprot.2017.10.010.

Chibisa, G.E., Gozho, G.N., van Kessel, A.G., Olkowski, A.A., Mutsvangwa, T., 2008. Effects of peripartum propylene glycol supplementation on nitrogen metabolism, body composition, and gene expression for the major protein degradation pathways in skeletal muscle in dairy cows. *Journal of Dairy Science* 91, 3512-3527. https://doi.org/10.3168/jds.2007-0920.

Chizzotti, M.L., Valadares, S.D., Valadares, R.F.D., Chizzotti, F.H.M., Tedeschi, L.O., 2008. Determination of creatinine excretion and evaluation of spot urine sampling in Holstein cattle. *Livestock Science* 113, 218-225.

Cornelius, C.E., Baker, N.F., Kaneko, J.J., Douglas, J.J., 1962. Distribution and turnover of iodine-131-tagged bovine albumin in normal and parasitized cattle. *American Journal of Veterinary Research* 23, 837-842.

Davis, T.A., Fiorotto, M.L., 2005. Regulation of skeletal muscle protein metabolism in growing animals. In: Burrin, D.G., Mersmann, H.J. (Eds.), Biology of Metabolism in Growing Animals, Vol. 3. Elsevier, pp. 35-68.

Detmann, E., Valente, E.E.L., Batista, E.D., Huhtanen, P., 2014. An evaluation of performance and efficiency of nitrogen utilization in cattle fed tropical grass pastures with supplementation. *Livestock Science* 162, 141-153. https://doi.org/10.1016/j.livsci.2014.01.029.

Doepel, L., Lapierre, H., Kennelly, J.J., 2002. Peripartum performance and metabolism of dairy cows in response to prepartum energy and protein intake. *Journal of Dairy Science* 85, 2315-2334.

Drackley, J.K., 1999. Biology of dairy cows during the transition period: The final frontier? *Journal of Dairy Science* 82, 2259-2273. https://doi.org/10.3168/jds.S0022-0302(99)75474-3.

Drackley, J.K., Cardoso, F.C., 2014. Prepartum and postpartum nutritional management to optimize fertility in high-yielding dairy cows in confined TMR systems. *Animal* 8 (Suppl. 1), 5-14. https://doi.org/10.1017/S1751731114000731.

Edmonson, A.J., Lean, I.J., Weaver, L.D., Farver, T., Webster, G., 1989. A body condition scoring chart for Holstein dairy cows. *Journal of Dairy Science* 72, 68-78.

Edouard, E., Hassouna, M., Robin, P., Faverdin, P. 2016. Low degradable protein supply to increase nitrogen efficiency in lactating dairy cows and reduce environmental impacts at barn level. *Animal* 10, 212-220. https://hal.science/hal-01209292.

FASS., 2010. Guide for the Care and Use of Agricultural Animals in Research and Teaching, 3rd ed. Federation of Animal Science Societies, Champaign, IL.

Faverdin, P., Verite, R., 1998. Utilisation de la teneur en uree du lair indicateur de la nutrition protcique et des rejets azotes chez la vache hitiere, *Rencontres Rccherches Ruminants* 5, 209-215.

Guinard, J., Rulquin, H., 1994. Effects of graded amounts of duodenal infusions of lysine on the mammary uptake of major milk precursors in dairy cows. *Journal of Dairy Science* 77, 3565e76.

Hayirli, A., Grummer, R.R., Nordheim, E.E., Crump, E., 2002. Animal and dietary factors affecting feed intake during the prefresh transition period in Holsteins. *Journal of Dairy Science* 85, 3430-3443. https://doi.org/10.3168/jds.S0022-0302(02)74431-7.

Houweling, M., Van der Drift, G.A., Jorritsma, R., Tielens, A.G.M., 2012. Technical note: Quantification of plasma 1- and 3-methylhistidine in dairy cows by high-performance liquid chromatography tandem mass spectrometry. *Journal of Dairy Science* 95, 3125-3130.

Huyler, M.T., Kincaid, R.L., Dostal, D.F., 1999. Metabolic and yield responses of multiparous Holstein cows to prepartum rumen undegradable protein. *Journal of Dairy Science* 82, 527-536. https://doi.org/10.3168/jds.S0022-0302(99)75264-1.

Kokkonen, T., Taponen, J., Anttila, T., Syrjälä-Qvist, L., Delavaud, C., Chilliard, Y., Tuori, M., Tesfa, A.T., 2005. Effect of body fatness and glucogenic supplement on lipid and protein mobilization and plasma leptin in dairy cows. *Journal of Dairy Science* 88, 1127-1141. https://doi.org/10.3168/jds.S0022-0302(05)72779-X.

Komaragiri, M.V.S., Casper, D.P., Erdman, R.A., 1998. Factors affecting body tissue mobilization in early lactation dairy cows. 2. Effect of dietary fat on mobilization of body fat and protein. *Journal of Dairy Science* 81, 169-175.

Lean, I.J., Van Saun, R., Degaris, P.J., 2013. Energy and protein nutrition management of transition dairy cows. *Veterinary Clinics of North America: Food Animal Practice* 29,337-366. https://doi.org/10.1016/j.cvfa.2013.03.005.

Lee, C. Morris, D.L., Dieter, P.A., 2019. Validating and optimizing spot sampling of urine to estimate urine output with creatinine as a marker in dairy cows. *Journal of Dairy Science.* 102, 236-245.

Lin, X., Li, S., Zou, Y., Zhao, F., Liu, J., Liu, H., 2018. Lysine stimulates protein synthesis by promoting the expression of ATB0, + and activating the mTOR pathway in bovine mammary epithelial cells. *Journal of Nutrition* 148,1426e33.

Linder, D.R., Tigemeyer, E.C., Olson, K.C., Anderson, D.E., 2014. Effects of gestation and lactation on forage intake, digestion, and passage rates of primiparous beef heifers and multiparous beef cows. *Journal of Animal Science* 92, 2141-2151. https://doi.org/10.2527/jas.2013-6813.

Megahed, A.A., Hiew, M.W.H., Ragland, D., Constable, P.D., 2019. Changes in skeletal muscle thickness and echogenicity and plasma creatinine concentration as indicators of protein and intramuscular fat mobilization in periparturient dairy cows. *Journal of Dairy Science* 102, 5550-5565. https://doi.org/10.3168/jds.2018-15063.

Moharrery, A., Das, T.K., 2002. Correlation between microbial enzyme activities in the rumen fluid of sheep under different treatments. *Reproduction Nutrition Development* 41, 513-529.

NASEM (National Academies of Sciences, Engineering, and Medicine), 2021. Nutrient Requirements of Dairy Cattle. 8th Rev. ed. National Academy Press, Washington, DC, USA.

Nishizawa, N., Toyoda, Y., Noguchi, T., Hareyama, S., Itabashi, H., Funabiki, R., 1979. Nτ-Methyl histidine content of organs and tissues of cattle in an attempt to estimate fractional catabolic and synthetic rates of myofibrillar proteins of skeletal muscle during growth by measuring urinary output of Nτ-methyl histidine. *British Journal of Nutrition* 42, 247-252. https://doi.org/10.1079/BJN19790111.

NRC, 1988. Nutrient Requirements of Dairy Cattle. 6th ed. National Academy Press Washington, DC, USA.

NRC, 2001. Nutrient Requirements of Dairy Cattle. 7th ed. National Academy Press Washington, DC, USA.

Pires, J.A., Delavaud, A.C., Faulconnier, Y., Pomies, D., Chilliard, Y., 2013. Effects of body condition score at calving on indicators of fat and protein mobilization of periparturient Holstein-Friesian cows. *Journal of Dairy Science* 96, 6423-6439.

Sadri, H., Ghaffari, M.H., Sauerwein, H., 2023. Invited review: Muscle protein breakdown and its assessment in periparturient dairy cows. *Journal of Dairy Science* 106, 822-842. https://doi.org/10.3168/jds.2022-22068.

Santos, J. E., DePeters, E.J., Jardon, P.W., Huber, J.T., 2001. Effect of prepartum dietary protein level on performance of primigravid and multiparous Holstein dairy cows. *Journal of Dairy Science* 84, 213-224. https://doi.org/10.3168/jds.S0022-0302(01)74471-2.

Siachos, N., Tsiamadis, V., Oikonomou, G., Panousis, N., Banos, G., Sampsonidis, I., Kalogiannis, S., Arsenos, G., Valergakis, G. E., 2024. Variation in protein metabolism biomarkers during the transition period and associations with health, colostrum quality, reproduction, and milk production traits in Holstein cows. *Journal of Dairy Science* 107, 4056-4074. https://doi.org/10.3168/jds.2023-24168.

Stanley, T.A., Cochran, R.C., Vanzat, E.S., Harmon, D., Corah, L., 1993. Periparturient changes in intake, ruminal capacity, and digestive characteristics in beef cows consuming alfalfa hay. *Journal of Animal Science* 71, 788-795. https://doi.org/10.2527/1993.713788x.

Tee, A.R., 2018. The target of rapamycin and mechanisms of cell growth. *International Journal of Molecular Science* 19, 880-892. https://doi.org/10.3390/ijms19030880.

Titgemeyer, E.C., Merchen, N.R., Berger, L.L., 1989. Evaluation of soybean meal, corn gluten meal, blood meal and fish meal as sources of nitrogen and amino acids disappearing from the small intestine of steers. *Journal of Animal Science* 67, 262e75.

Topps, J.H., Elliott, R.C., 1965. Relationship between concentrations of ruminal nucleic acids and excretion of purine derivatives by sheep. *Nature* 205, 498-499.

Valadares, R.F., Broderick, D.G.A., Valadares, S.C., Clayton, M.K., 1999. Effect of replacing alfalfa silage with high moisture corn on ruminal protein synthesis estimated from excretion of total purine derivatives. *Journal of Dairy Science* 82, 2686-2696.

Van der Drift, S.G.A., Houweling, M., Schonewille, J.T., Tielens, A.G.M., Jorritsma, R., 2012. Protein and fat mobilization and associations with serum β-hydroxybutyrate concentrations in dairy cows. *Journal of Dairy Science* 95, 4911-4920.

Van Knegsel, A.T., Van Den Brand, H., Dijkstra, J., Van Straalen, W.M., Heetkamp, M.J.W., Tamminga, S., Kemp, B., 2007. Dietary energy source in dairy cows in early lactation: Energy partitioning and milk composition. *Journal of Dairy Science* 90,1467-1476.

Van Soest, P.J., Robertson, J.B., Lewis, B.A., 1991. Methods for dietary fiber, neutral detergent fiber, and non-starch polysaccharides in relation to animal nutrition. *Journal of Dairy Science* 74, 3583-3597.