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Protein requirements for pregnant dairy cows

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ABSTRACT

We aimed to estimate the protein requirements of pregnant Holstein × Gyr cows. A total of 61 Holstein × Gyr (HG) cows were used, with an average initial body weight (BW) of 480 ± 10.1 kg and age 5 ± 0.5 yr. Cows were divided into 3 groups: pregnant (n = 43), nonpregnant (n = 12), and baseline (n = 6). Baseline animals were slaughtered before starting the experiment to estimate the initial body composition of the remaining animals. Pregnant and nonpregnant cows received 2 diets: maintenance and ad libitum. Pregnant cows were slaughtered at 139, 199, 241, and 268 gestation days (GD). First, we used data only from nonpregnant cows to determine the requirements for maintenance and growth in adult cows. The requirements of metabolizable protein for maintenance (MP_m; grams of empty metabolic BW [$EBW^{0.75}$] per day) were estimated using a linear regression between the metabolizable protein intake (MPI, g/d) and average daily gain (g/d), and the MP_m was defined as the intercept divided by the average metabolic BW. Net protein requirements for gain (NP_g; g/d) were estimated by the first derivative of the allometric equation between final crude protein in the body (kg) and the final empty BW (EBW; kg). The efficiency of use of metabolizable protein for gain (k) was calculated from the regression between the retained protein (g $EBW^{0.75}$/d) and the MPI (g $EBW^{0.75}$/d), and k was the slope of this regression. The MPI was estimated by combining microbial protein synthesis (purine derivatives) with the digestible rumen undegradable protein [(total protein intake − rumen-degradable protein) × intestinal digestibility]. Second, an exponential model was used to fit the protein accumulation in the gestational components in the function of GD. The first derivative of that model was considered the net requirement for gestation (NP_{gest}). The efficiency of protein utilization for gestation (k_{gest}) was calculated by the iterative method using the equation $\Delta = MPI - (MP_m + NP_g/k_g + NP_{gest}/k_{gest})$, where k_g is efficiency of protein utilization for gain. The iteration was performed aiming at a zero deviation between observed MPI and metabolizable protein (MP) estimated by the requirements determined herein. We obtained a value of 3.88 g $EBW^{0.75}$/d for MP_m. The estimation of NP_g can be calculated according to the following equation: $NP_g = 0.716 \times (EBW_{open}{}^{-0.308}) \times EBG_c$, where EBW_{open} is the EBW (kg) for nonpregnant animals and EBG_c is the empty body gain (kg/d) corrected for the gestational component. The k was determined as 0.347. The NP_{gest} requirements were determined as NP_{gest} (g/d) = $0.0008722 \times \exp^{(0.01784 \times GD)} \times$ (calf weight/35). The k_{gest} was 0.625. It is important to highlight that different methods of MP estimates should not be mixed and that the proposed method requires the estimation of microbial protein (estimated via urinary estimates), which might limit practical application. In conclusion, new studies should be conducted to validate our results and the methodology adopted to determine protein requirements for pregnancy in dairy cows. Due to the pattern of protein accumulation in the gestational components, we suggest an exponential model to describe protein requirements for pregnancy for dairy cows.
Key words: fetal growth, fetus, gestation

INTRODUCTION

Worldwide, diets for dairy cattle are formulated based on international systems such as AFRC (1993), CSIRO (2007), INRA (2018), NASEM (2021), and NRC (2001). For example, the eighth revised edition of the Nutrient Requirements of Dairy Cattle (NASEM, 2021) describes a nutrient requirement as the daily amount of a certain nutrient necessary to meet a healthy cow's needs for maintenance, activity, growth, reproduction, and lactation without changing the body reserves or physiological status.

The international literature contains only 3 major publications reporting protein requirements for pregnant cows: (1) Bell et al. (1995) with dairy cows, (2)

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Ferrell et al. (1976) with beef heifers, and (3) BR-CORTE (Valadares Filho et al., 2016) with beef Nellore cows. The first 2 studies (Ferrell et al., 1976; Bell et al., 1995) were the basis for establishing requirements for pregnancy in all major requirements systems. Given the intensive selection for milk production over the past 50 yr, research suggests that modern dairy cows have greater metabolic rates than they had in the past (NASEM, 2021). However, the eighth revised edition of NASEM (2021) used the previous edition as the starting point (same database) but with new statistical tools to estimate requirements for pregnancy according to gestation days (**GD**) between 12 and 280 d (although the requirements for maintenance were estimated based on new data). The INRA Feeding System for Ruminants (INRA, 2018) was also developed based on an old database (Ferrell et al., 1976); however, a new statistical approach was applied to calculate requirements for pregnant cows. Nonetheless, it is clear that we have based all our requirements for gestation on only a few studies, and new data are needed to either validate the new recommendations (INRA or NASEM) or bring a new perspective to the topic.

In the case of this study, we used Holstein × Gyr cows as a model of high-producing cows adapted to tropical climates. The calf's weight at birth is considered a determinant factor in the requirements for gestation. Therefore, a calf birth weight correction (calf birth weight/45) is added to all those systems by multiplying the net requirement for pregnancy (including this study) and should control most of those confounding effects, making it possible to use data from different breeds to determine requirements with reasonable accuracy.

Nevertheless, the NASEM (2021) was only recently published, and the NRC (2001) is still the most widespread system. Identifying the nutritional requirements of pregnant cows aims to ensure an adequate nutrient supply for the growth and development of the fetus, optimize the BCS at calving, and provide adequate nutrients for milk production in early lactation. Thus, the objective of the present study was to estimate the protein requirements for maintenance, body gain, and gestation of Holstein × Gyr (**HG**) crossbred cows.

MATERIALS AND METHODS

This study was conducted at Universidade Federal de Viçosa (Viçosa, MG, Brazil), following the standard procedures for humane animal care and handling according to the university's guidelines (CEUAP/UFV, protocol number: 11/2013). All animals originated from the same farm (Embrapa Gado de Leite, Minas Gerais, Brazil) and management and formed a contemporary group. The sample size was calculated to detect a 15% difference in body protein (kg) between ad libitum and maintenance diets (14.5% CV; Valadares Filho et al., 2016), with 95% confidence at 90% power (Ryan, 2013).

Animals and Management

A total of 61 Holstein × Gyr cows were used, with an average initial BW of 480 ± 10.1 kg and age 5 ± 0.5 yr. Briefly, cows were monitored for estrus every 12 h and artificially inseminated using semen from a single Holstein bull 12 h after estrus. The pregnancy was verified on d 60 of gestation, and cows entered the experiment. The cows were randomly assigned into 3 groups: pregnant (n = 43), nonpregnant (n = 12), and baseline (n = 6). First, all 61 cows underwent an adaptation period of 14 d. The adaptation period was necessary to standardize all animals and management conditions. After adaptation, all baseline animals (open cows) were slaughtered to compose the reference group, which is essential in comparative slaughter trials, once their body composition was used to estimate initial body composition and initial BW of the remaining cows. After the slaughter of baseline animals, pregnant (43) and nonpregnant (12) cows were randomly allocated into 2 different diets: maintenance (**MA**; n = 29) or ad libitum (**AD**; n = 26). For the MA group, the DMI was considered to be 1.15% of BW, as calculated by Duarte et al. (2013). The cows were weighed every 28 d after a 16-h fast to obtain the shrunk BW. The shrunk BW was used to adjust the feed intake of the MA treatment. To evaluate the effects of days of gestation, pregnant and nonpregnant animals were slaughtered at 140, 200, 240, and 270 GD, as described by (Rotta et al., 2015a,b,c) and illustrated in Figure 1 according to Sguizzato et al. (2020b).

Cows were housed in individual pens measuring 30 m^2, equipped with individual feed bunks and an automatic water system. All cows had ad libitum access to water. The diet (Table 1) was based on corn silage and concentrate, with a ratio of 93:7 DM basis as a TMR containing 11.1% CP and 49.7% NDF. Cows were fed twice daily, with 60% offered in the morning and 40% in the afternoon feeding. The amounts of corn silage, concentrate, and orts were recorded daily. Corn silage and orts were sampled daily and stored at −20°C until analysis. To allow ad libitum feeding, cows from AD treatment had their diets adjusted to allow approximately 5% orts daily on an as-fed basis.

Fecal Measurements, Urine, and Harvest

The study had 6 periods of spot fecal and urine collection within each period of 28 d. Feces were collected

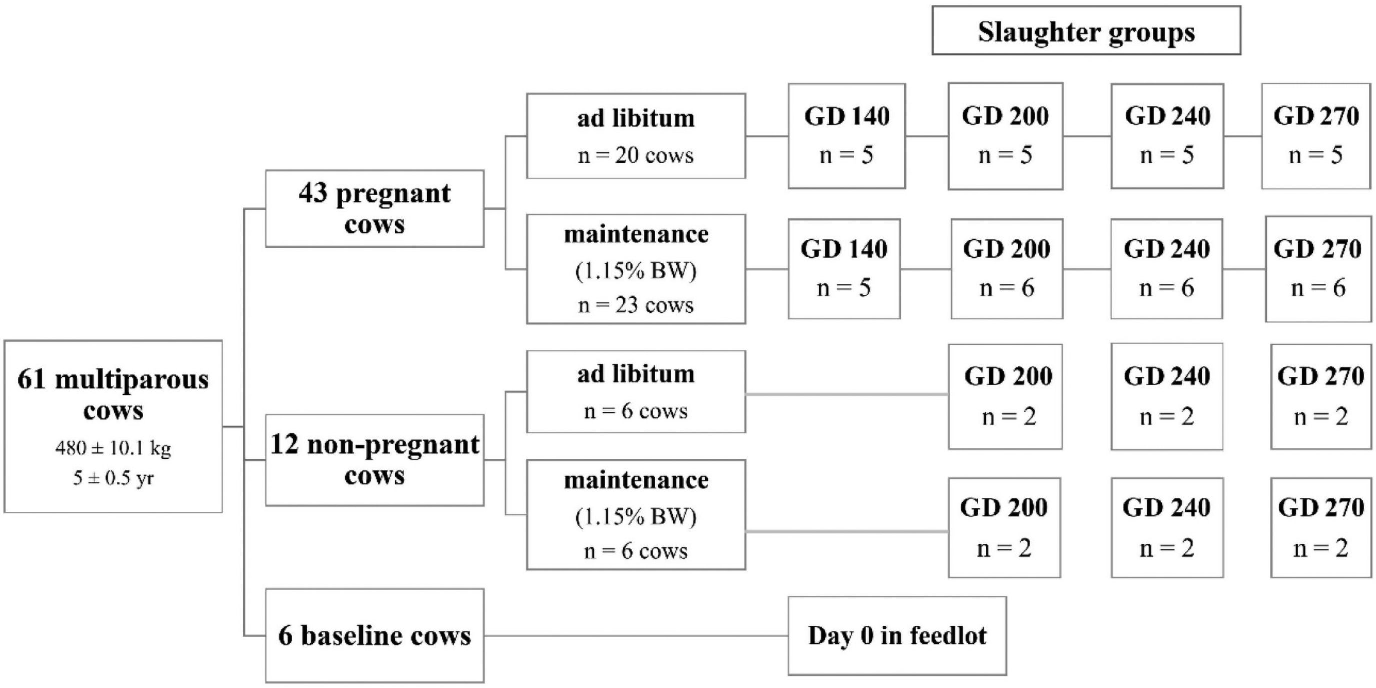

Figure 1. Experimental scheme, feeding regimens, and slaughter groups (from Sguizzato et al., 2020b). GD = gestation days.

during the last 5 d of each period. Fecal collections were performed at 0600, 0900, 1200, 1500, and 1800 h, with 1 collection per day. The fecal samples contained approximately 200 g each. They were collected by rectal stimulation or immediately after the animal defecated to avoid feces contamination. The samples were dried in a forced-air oven at 55°C for 72 h and ground in a 1-mm screen (Wiley mill; A. H. Thomas, Philadelphia, PA). A composite fecal sample from each animal was obtained at the end of each period, using 15 g of the dried and ground sample per day. Urine collections were performed at 0600 and 1500 h on the first and fourth days of fecal collection, respectively, by vulva stimulation. After collection, urine samples were acidified to a pH <4.0 with concentrated sulfuric acid and then frozen at −20°C for further analyses of N, urea, allantoin, creatinine, and uric acid.

Before slaughter, feed was withheld overnight, but cows had free access to water. The slaughter was performed at 140, 200, 240, and 270 GD by a captive bolt stunner, followed by exsanguination. After exsanguination, the gravid uterus was immediately collected through sectioning at the height of the cervix and weighed. The gravid uterus was dissected into the fetus, fetal membranes, uterus, and fetal fluids, and each component was ground and individually sampled. The mammary gland was also entirely sectioned and ground. For further analysis, the pregnant uterus and mammary gland samples were stored at −80°C.

The carcass of each animal was divided into 2 half carcasses. They were weighted to determine hot carcass yield and then allocated in a cold chamber at 4°C for 24 h. Posteriorly, the carcasses were weighted to determine cold carcass yield. In addition, to compose the non-carcass sample, the 4 stomach chambers and small and large intestines were washed after slaughter and added to internal organs, head, tail, feet, trimmings, hide, and blood. Next, carcass and non-carcass components were ground and homogenized separately using an industrial grinder after passing through a shredder knife box. Then a sample of each carcass and non-carcass was taken for further analyses and finally composed cow's tissue sample.

Laboratory Analyses

Corn silage, concentrate ingredients, and feces were analyzed for DM, OM, and N concentrations (AOAC International, 2000; methods number 934.01 for DM, 930.05 for OM, and 981.10 for N). The NDF was estimated according to Mertens et al. (2002) without the addition of sodium sulfite and with the addition of thermostable α-amylase to the detergent (Ankom Technology Corp., Fairport, NY). Samples of carcass, non-

Table 1. Ingredients and chemical composition of the experimental diet, % DM except where otherwise indicated[1]

Item	% DM
Ingredient	
Corn silage	93.0
Cottonseed meal	5.0
Limestone	0.5
Salt	0.5
Urea	0.9
Ammonium sulfate	0.1
Mineral mix[2]	0.02
Chemical composition	
DM, %	37.6
OM, %	92.9
CP, %	11.1
MP, g/kg DM	70.7
AA,[3] % MP	
Arg	5.5
His	2.2
Ile	6.2
Leu	9.1
Lys	8.0
Met	2.4
Phe	6.0
Thr	5.7
Trp	1.3
Val	6.4
NDF, % DM	49.7
Ether extract	3.7
Starch	23.2
NFC	28.4
Indigestible NDF	15.7
Mineral, g/kg DM	
Calcium	3.9
Phosphorus	1.9
Magnesium	1.6
Potassium	11.7
Sodium	2.2
Sulfur	2.2

[1]Adapted from Rotta et al., 2015a,b,c.

[2]Mineral mix composition = 29.2 g/kg calcium, 0.70 g/kg phosphorus, 2.11 g/kg magnesium, 0.89 g/kg potassium, 0.31 g/kg sodium, 63.5 g/kg sulfur, 348 mg/kg cobalt, 2.56 mg/kg chromium, 3,296 mg/kg copper, 2,088 mg/kg iron, 4,673 mg/kg manganese, 7,817 mg/kg zinc, and 318 mg/kg selenium.

[3]Based on NASEM (2021) AA profile of feed ingredients.

carcass, mammary gland, uterus, placenta, fetal fluids, and fetus were analyzed for DM (AOAC International, 2000; method 934.01), CP (AOAC International, 2000; method 981.10), and ether extract (AOAC International, 2000; method 945.16).

Fecal excretion was estimated using undigestible NDF as an internal marker. Samples of feces and feeds were ground through a 2-mm screen. A 1.5-g sample was weighed in each pre-weighed polyester bag with a pore size of 12 μm and a pore area of 6% of the total surface (Saatifil PES 12/6, Saatitech S.p.A., Veniano, Como, Italy). The incubation lasted 288 h and was performed in 2 cannulated bulls fed a diet of 50% corn silage and 50% concentrate based on DM at the MA level. After incubation, bags were washed, dried at 55°C for 48 h, and weighed. Residues were analyzed for NDF using an Ankom 200/220 fiber analyzer (Ankom Technology Corp., Fairport, NY).

Allantoin, creatinine, and uric acid were analyzed via HPLC (Agilent 1100 series, Agilent Technologies, Waldbronn, Germany) as previously described by Czauderna and Kowalczyk (2000), with modifications by George et al. (2006). Carcass, non-carcass, mammary gland, uterus, placenta, fetal fluid, and fetus samples were analyzed for DM (AOAC International, 2000; method 934.01), CP (AOAC International, 2000; method 981.10), and ether extract (AOAC International, 2000; method 945.16). Energy content was estimated based on protein and ether extract contents, as proposed by ARC (1980).

Calculations and Estimated Equations

To estimate the excretion of purine derivatives, equations previously described by Verbic et al. (1990), Chen and Gomes (1992), and Chen et al. (1995) were used. The supply of microbial nitrogen estimated through the excretion of purine derivatives was estimated from 4 intermediate steps: calculation of total purine derivatives, daily excretion of nitrogen, purines absorbed daily, and, finally, supply of microbial N. More details on calculations and equations used are available in Rotta et al. (2015b).

Microbial crude protein (**MCP**) was estimated via the following equation:

$$\text{MCP} = \text{microbial N} \times 6.25, \quad [1]$$

where MCP and microbial are N measured in grams per day.

As this study was conducted with low dietary protein levels (11.1%), recent publications (NASEM, 2016) have shown that the N loss via the rumen wall when there is a conversion from RDP to microbial protein is very close to the N recycled via the rumen wall; thus, we considered RDP to be equal to microbial protein N. Therefore, the RUP intake was estimated as the difference between the CP intake and RDP.

The MP intake (**MPI**) was calculated considering that 80% of microbial protein is true protein and 80% intestinal digestibility for MCP. Additionally, we considered the proportion of ingredients in the diet and their respective intestinal digestibility to calculate an average RUP digestibility of 81%:

$$\text{MPI} = (\text{RDP} \times 64\% + \text{RUP} \times 81\%), \quad [2]$$

where MPI, RDP, and RUP are measured in grams per day. Proteins originating from bacteria and protozoa were considered to have 80% of true protein and 80% digestibility; thus, 64% was used from RDP to MPI. The RUP was considered to contain 100% true protein with 81% digestibility, based on NASEM (2021).

Finally, it is essential to highlight that, as we used many assumptions and some calculations based on purine derivatives to determine MPI, we do have errors associated with the variable present in the x-axis (i.e., Eq. 2). However, when trying to use only equations already present in the literature to determine MPI, we got results that were not biologically valid and decided to keep this methodology even though acknowledging that it adds a new layer of errors in our prediction models.

The following model calculates the relationship between BW and empty BW (**EBW**). We also tested the effect of GD on the model parameters:

$$\text{EBW or EBG} = \beta_0 + \beta_1 \times \text{BW or ADG}, \quad [3]$$

where β_0 and β_1 = regression parameters; **EBG** = empty body gain (kg/d); and BW is measured in kilograms.

Requirements for Maintenance. The MP requirements for maintenance (**MP**$_{\mathbf{m}}$, g/kg of metabolic BW [**BW**$^{\mathbf{0.75}}$]) were estimated using exclusively nonpregnant cows, as proposed by Wilkerson et al. (1993). We used a linear regression between MPI (kg/d) and EBG (kg/d), with subsequent division of the intercept by the average metabolic BW:

$$\text{MPI} = \beta_0 + \beta_1 \times \text{EBG}, \quad [4]$$

where β_0 and β_1 are regression parameters.

The EBG was obtained from the EBW at the end of the trial minus EBW at the beginning of the trial divided by days in the experiment. The final EBW was obtained by weighing all body components after washing the gastrointestinal tract during slaughter. The initial EBW was determined based on the relationship between EBW and BW of the baseline animals. The user will be able to estimate the EBW based on the equations proposed in this study (Eq. 3):

$$\text{MP}_{\text{m}} = \frac{\beta_0}{\text{EBW}^{0.75}}, \quad [5]$$

where MP_{m} is measured in grams per kilogram of empty metabolic BW (**EBW**$^{\mathbf{0.75}}$), β_0 is the intercept of Eq. 5, and $\text{EBW}^{0.75}$ is the mean average metabolic BW of the nonpregnant cows used in this study (94.33 kg).

Requirements for Gain. The body protein content (kg) in EBW of nonpregnant animals (**EBW**$_{\mathbf{open}}$) was estimated using the allometric model according to Fortin et al. (1980):

$$\text{BCP} = \beta_0 \times (\text{EBW}_{\text{f}}^{\ \beta 1}), \quad [6]$$

where **BCP** is the body protein content in final EBW for nonpregnant tissues (kg), **EBW**$_{\mathbf{f}}$ is the final EBW (kg), and β_0 and β_1 are regression parameters. For this equation, we used BW and CP body composition of nonpregnant and baseline animals.

The net protein requirements for gain (**NP**$_{\mathbf{g}}$, g/d) were estimated by the first derivative of the previous equation multiplied by EBG, as follows:

$$\text{NP}_{\text{g}} = \beta_0 \times \beta_1 \times \text{EBW}^{\beta 1-1} \times \text{EBG}_{\text{c}}, \quad [7]$$

where EBW is measured in kilograms, **EBG**$_{\mathbf{c}}$ is the EBG corrected for the gestational (**GEST**) components gain (accretion in the udder, uterus, and all the other components of the gravid uterus due to pregnancy). The description of how we calculated GEST and EBG_{c} will be more fully described later in the Requirements for Gestation section. Finally, β_0 and β_1 are regression parameters.

The efficiency of use of metabolizable protein for gain (***k***) was calculated from the regression between the retained protein (g $\text{EBW}^{0.75}$/d) and the MPI (g $\text{EBW}^{0.75}$/d):

$$\text{RP} = \beta_0 + \beta_1 \times \text{MPI}, \quad [8]$$

where **RP** is the retained protein of nonpregnant cows (g $\text{EBW}^{0.75}$/d), and β_1 is admitted as k_{g} (efficiency of protein utilization for gain).

The MP requirements for gain (**MP**$_{\mathbf{g}}$, g/d) were estimated by dividing NP_{g} by k.

Requirements for Gestation. To estimate the requirements for gestation, the balance of gestation components and days of gestation were considered. The balance of gestation components was calculated as the sum of the final and initial protein contents of the gravid uterus and the difference between the final and initial protein contents of the udder in kilograms. To predict initial gestation components, an allometric regression (based on EBW) was estimated in the function of final gestation components and final EBW, using baseline and nonpregnant cows (Sguizzato et al., 2020a). As every cow has GEST, this equation was established to determine the weight and protein of GEST in pregnant cows if they were not pregnant (**GEST**$_{\mathbf{open}}$, Eq. 9, Figure 2)—in other words, the GEST weight and protein that do not contribute to pregnancy requirements:

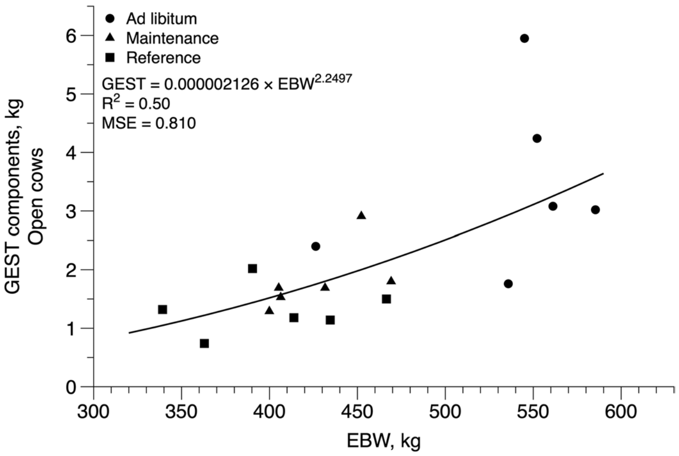

Figure 2. Gestational components (GEST: uterus + placenta + fetal fluids + mammary gland) weight (kg of DM) in open Holstein × Gyr dairy cows fed ad libitum or maintenance diets, or harvested at the beginning of the experiment (reference), according to empty body weight (EBW, kg). MSE = mean squared error.

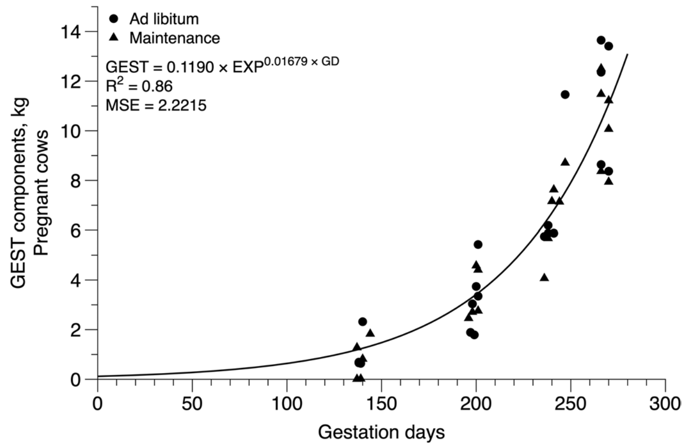

Figure 3. Gestational components (GEST: uterus + placenta + fetus + fetal fluids + mammary gland) weight (kg of DM) in pregnant Holstein × Gyr dairy cows fed ad libitum or maintenance diets according to gestation days. MSE = mean squared error.

$$\mathrm{GEST_{open}} = 0.000002126_{\pm 0.0000000001} \times \mathrm{EBW}^{2.2497_{\pm 0.01016}}$$

$$(\mathrm{R}^2 = 0.501;\ \mathrm{RMSE} = 0.900), \quad [9]$$

where $\mathrm{GEST_{open}}$ and EBW are measured in kilograms; **RMSE** = root mean squared error.

Then, we used an exponential model with 43 pregnant animals to estimate protein accumulation in GEST of pregnant cows (**$\mathrm{GEST_{preg}}$**, Eq. 10, Figure 3):

$$\mathrm{GEST_{preg}} = \left(0.1190_{\pm 0.03869}\right) \times \exp^{\left(0.01679_{\pm 0.001274}\right) \times \mathrm{GD}}$$

$$(\mathrm{R}^2 = 0.862;\ \mathrm{RMSE} = 1.490), \quad [10]$$

where $\mathrm{GEST_{preg}}$ and EBW are measured in kilograms, GD in days.

We used the balance of GEST ($\mathrm{GEST_{preg}} - \mathrm{GEST_{open}}$) and their respective protein compositions (based on GD) to estimate protein requirements for gestation. Then, among simple exponential, double exponential, and power functions, the simple exponential model was selected to fit the gestation component (protein) as a function of GD:

$$\text{Simple exponential model, } \mathrm{Y} = \beta_0 \times \exp^{(\beta_1 \times \mathrm{GD})}, \quad [11]$$

where β_0 and β_1 are equation parameters.

To assess the best fit, the Akaike information criterion was used. This method demonstrates, within a group of preselected models, the one with the lowest Kullback-Leibler divergence. The lowest Akaike information criterion value found for a model represents the best fit.

Finally, the efficiency of protein utilization for gestation (**k_{gest}**) was calculated by the iterative method using the following equation:

$$\Delta = \mathrm{MPI} - \left(\mathrm{MP_m} + \frac{\mathrm{NP_g}}{k} + \frac{\mathrm{NP_{gest}}}{k_{gest}}\right), \quad [12]$$

where MPI, $\mathrm{MP_m}$, and $\mathrm{NP_g}$ are measured in grams per day; k and k_{gest} are measured as percentages; $\mathrm{NP_{gest}}$ = net protein for gestation components (g/d).

The iteration was performed aiming at a zero deviation between MPI and the other MP requirements values. The components (MPI, $\mathrm{MP_m}$, $\mathrm{NP_g}$, k, and $\mathrm{NP_{gest}}$) were calculated according to previously described models.

A summary of all equations used in this study to estimate the protein requirements for gestation is listed in Table 2. For all estimations of requirements for protein and gain in pregnant animals (see Excel file Supplemental File S1, https://figshare.com/s/98922f1d35d77497c9ef), EBW was converted to $\mathrm{EBW_{open}}$ (kg) as EBW − ($\mathrm{GEST_{preg}} - \mathrm{GEST_{open}}$). Thus, as mentioned before, the GEST components in an open cow are used for maintenance and gain requirements, and only GEST components exclusively due to pregnancy were used to estimate requirements for gestation.

Statistical Analyses

The linear regression parameters were estimated using PROC MIXED in SAS (9.4; SAS Institute Inc., Cary,

Table 2. Summary of prediction equations and protein requirements estimations for pregnant cows[1]

Item	Equation	Unit	Description
Intermediate calculations			
MCP	Microbial N × 6.25	g/d	Microbial crude protein
EBW	BW × 0.8737	kg	Empty BW, GD ≤200
EBW	BW × 0.9041	kg	Empty BW, GD >200
RDP	MCP × 1	g/d	Rumen-degradable protein
RUP	CP intake − RDP	g/d	Rumen-undegradable protein
MPI	RDP × 64% + RUP × 81%	g/d	MP intake
EBG	$(EBW_f - EBW_i)$/days	kg/d	Empty body gain
EBW_{open}	$EBW - GEST_{preg} + GEST_{open}$	kg	Empty BW of pregnant cows discounting the tissue accumulation on GEST due to the gestation
$GEST_{preg}$	$0.1190 \times \exp^{(0.01679 \times GD)}$	kg	Estimated portion of the accumulation of GEST tissues during to the gestation
$GEST_{open}$	$0.000002126 \times (EBW^{2.2497})$	kg	Estimated portion of GEST that occur naturally, without the influence of gestation
EBG_c	$EBG \times (1 - 0.004497 \times \exp^{0.01198 \times GD})$	kg/d	Empty body gain discounting weight of gestation components
GEST	Uterus + fetal fluids + fetus + placenta + mammary gland	kg	Gestation components
Protein requirements			
MP_m	$3.88 \times EBW^{0.75}$	g/d	MP for maintenance
NP_g	$0.7160 \times (EBW_{open}{}^{-0.3081}) \times EBG_c$	g/d	Net protein for gain
NP_{gest}	$0.0008722 \times \exp^{(0.01784 \times GD)} \times$ (calf weight/35)	g/d	Net protein for gestation
k_{gest}	0.625	—	Efficiency of use of MP for gestation
MP_{gest}	NP_{gest}/k_{gest}	g/d	MP for gestation

[1]GD = gestation days; EBW_f = final EBW; EBW_i = initial EBW; $GEST_{preg}$ = GEST of pregnant cows; $GEST_{open}$ = GEST of nonpregnant cows.

NC). The effect of physiological status (pregnant or nonpregnant) was tested on both parameters, β_0 and β_1. The model included diet as a fixed effect. Estimates of the parameters of nonlinear regressions were adjusted using PROC NLMIXED of SAS (9.4). Student's t-test was used for all statistical analyses to compare the intakes (MA vs. AD), and all significances were declared when $P < 0.05$.

RESULTS AND DISCUSSION

Relationship Between BW and EBW

We found no effect of GD on the model intercept, and the intercept did not differ from zero ($P > 0.05$). However, an effect of GD was found for the slope of the relationship between BW and EBW. Thus, the following equations were described to estimate EBW:

$$\text{if GD} < 200,\ EBW = BW \times 0.8776 \pm 0.0053;$$

$$\text{if GD} > 200,\ BW = BW \times 0.9018 \pm 0.0048$$

$$(R^2 = 0.9700;\ RMSE = 15.569), \qquad [13]$$

where EBW and BW are measured in kilograms.

Considerations Regarding MP Intake

As mentioned before, we used some assumptions and calculations based on purine derivatives to determine MPI; thus, we have errors associated with the variable in the x-axis (i.e., Eq. 2), which is not recommended for prediction models. Additionally, despite the obtained findings, it is crucial to exercise caution when interpreting the results of microbial protein synthesis. The use of purine derivates as a means to estimate microbial protein synthesis presents inherent limitations. According to Hristov et al. (2019), the main concern regarding purines lies in the unequal ratios of purine to total N observed in protozoal and bacterial pools, in addition to the assumption that dietary purines undergo complete degradation in the rumen (Smith and Mcallan, 1970; McAllan and Smith, 1973). Furthermore, recent research has revealed that dietary purines may contribute between 13 and 33% of the purine flow in the duodenum of cattle, leading to an overestimation of microbial protein synthesis (Pérez et al., 1997; Vicente et al., 2004; Hristov et al., 2005). Therefore, it has been advised by Hristov et al. (2019) that absolute changes in microbial protein synthesis should not be calculated based on urinary purine derivatives. The authors acknowledge that the estimate of metabolizable protein, and thus microbial protein synthesis, is central to our study; nonetheless, for this study, it was not possible to determine microbial protein production using different techniques without creating an extremely stressful situation for our animals, which, in gestation and heat stress conditions, could lead to loss of pregnancy. Therefore, it is important to highlight that future stud-

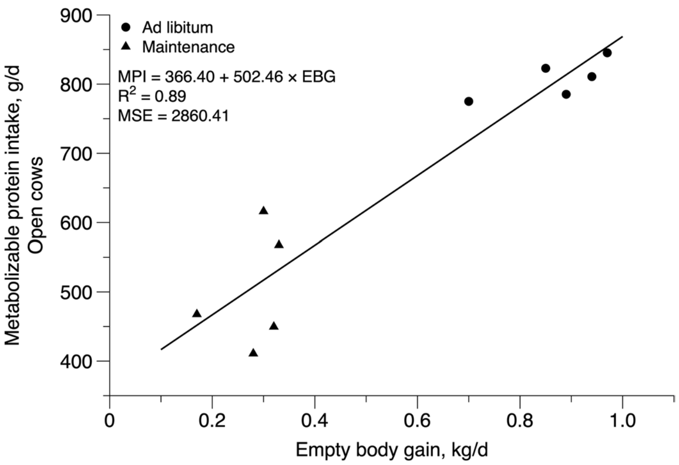

Figure 4. Metabolizable protein intake (MPI, g/d) of open Holstein × Gyr dairy cows fed ad libitum or maintenance diets according to empty body gain (EBG, kg/d). MSE = mean squared error.

ies should be conducted to validate the use of purine derivatives in pregnant cows and the validity of the results found herein.

To validate our estimates of MPI, we employed the NASEM (2021) model, incorporating our diets and feed composition. By applying this model, we successfully predicted the MPI values for AD and MA diets, resulting in estimates of 984 and 491 g/d, respectively. Notably, our predicted values closely align with our experimental data of 931 and 488 g/d, representing a marginal difference of only 5.4% and 0.3% for AD and MA diets, respectively. These findings significantly strengthen the reliability of our MPI estimates, affirming their congruence with the most up-to-date systems of nutrient requirements.

Metabolizable Protein Requirements for Maintenance

The MP_m was calculated by dividing the intercept of Eq. 4 (Figure 4) by the average $EBW^{0.75}$ of the cows used in the experiment (94.33 kg). Thus, the MP_m observed in this study was 3.88 g $EBW^{0.75}$/d:

$$\text{MPI (kg/d)} = 366.40 \pm 42.797 + 502.46 \pm 65.8149 \times \text{EBG (kg)}$$
$$(R^2 = 0.889; \text{RMSE} = 54.410), \quad [14]$$

where MPI and EBG are measured in kilograms per day.

The previous editions of the *Nutrient Requirements of Dairy Cattle* (i.e., NRC, 2001) reported the requirements of various nutrients without describing what the term "nutrient requirement" conceptually means (NASEM, 2021). However, the eighth revised edition (NASEM, 2021) describes nutrient requirement as the daily amount of a certain nutrient necessary to meet a healthy cow's needs for maintenance, activity, growth, reproduction, and lactation without changing the body reserves or the physiological status. This is the first time in the Dairy Requirement series that the meaning of "requirement" has been defined. Although evidence indicates that accurate requirement estimates may reduce the production costs and environmental impacts of dairy farming, differences in production systems (temperate vs. tropical) and breed composition might be factors that warrant investigation. For example, INRA (2018) and NASEM (2021) use the factorial approach to calculate MP_m and suggests a model that considers the sum of fecal endogenous N losses, endogenous urinary N losses, and scurf N losses. Considering the data of the present study, for a cow weighing 517 kg, with DMI of 9.71 kg/d, nondigestible OM of 2.33 kg, and efficiency of digestion of 67%, as suggested by this system, the MP_m estimated by INRA (2018) is 2.2 g $EBW^{0.75}$/d, which is 43% lower than the 3.88 g $EBW^{0.75}$/d presented in this study. Using the same condition and NASEM (2021) recommendations, this value would be 2.95 g $EBW^{0.75}$/d, which is 24% lower than our recommendations. The INRA feeding system for ruminants is a European system, but its data set was developed to be applied in different contexts, such as temperate, tropical, and Mediterranean conditions. It is well known that studies evaluating nutrient requirements for pregnancy are scarce due to the methodology applied, the comparative slaughter technique. In the comparative slaughter technique, at least 2 feeding levels (i.e., AD and MA) are required and involve the slaughter of baseline animals at the beginning of the experiment to develop the equations to estimate the initial BW and composition of the animals that will be slaughtered later (Tedeschi and Carstens, 2019). Therefore, the difference seen herein could be explained based on differences in methodologies, but also animals, climate conditions, breeds, and more. Further studies are still warranted to validate our results.

However, Castro et al. (2020) estimated values of 3.5 g $EBW^{0.75}$ of MP_m for crossbred heifers raised under tropical conditions. Although this value is lower than that observed in the present study, the value is much closer (only 10% lower), and the difference in the physiology status of the animals (heifers vs. nonpregnant cows) should be noted as a reason for that difference. Unfortunately, few studies have been conducted to estimate the maintenance requirements of protein in pregnant cows, and more studies are warranted to develop accurate equations to meet the requirements of a modern dairy cow (NASEM, 2021).

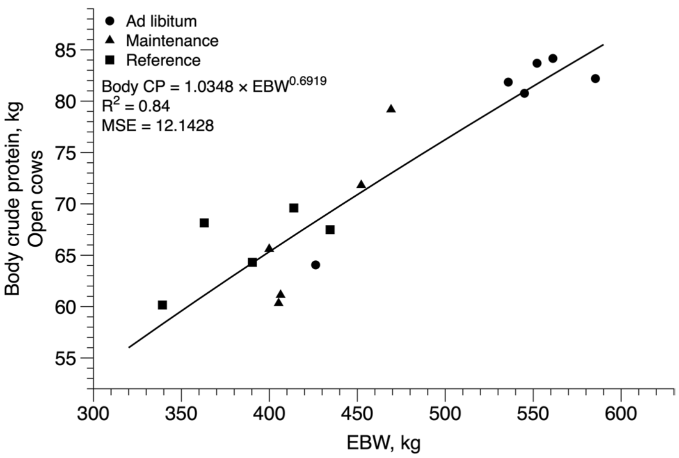

Figure 5. Body total CP (kg) of open Holstein × Gyr dairy cows fed ad libitum or maintenance diets, or harvested at the beginning of the experiment (reference), according to empty body weight (EBW, kg). MSE = mean squared error.

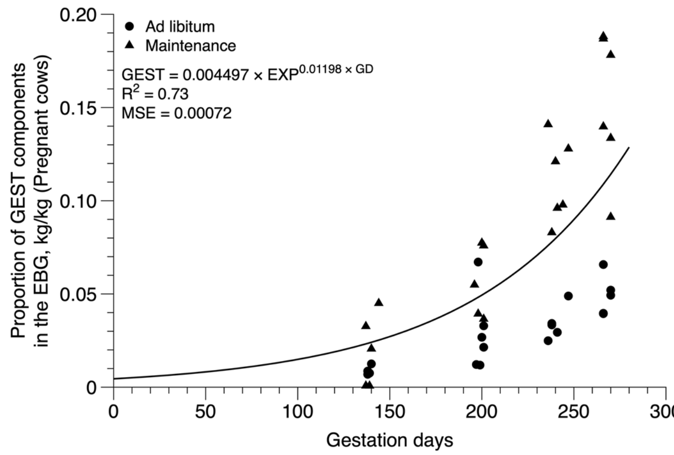

Figure 6. Proportion of gestational components (GEST: uterus + placenta + fetus + fetal fluids + mammary gland) in the empty body gain (EBG) of pregnant Holstein × Gyr dairy cows fed ad libitum or maintenance diets, according to gestation days. MSE = mean squared error.

Requirements for Gain

The body protein content can be estimated following the equation (Figure 5):

$$\mathrm{BCP} = 1.0348 \pm 0.2562 \times \left(\mathrm{EBW_f}^{0.6919_{\pm 0.03945}}\right)$$

$$(R^2 = 0.840;\ \mathrm{RMSE} = 3.485), \quad [15]$$

where BCP and $\mathrm{EBW_f}$ are measured in kilograms.

The $\mathrm{NP_g}$ was calculated using the first derivative of the equation of BCP multiplied by $\mathrm{EBG_c}$. In the present study, we used the $\mathrm{EBG_c}$ for the final calculation of the requirement for gain (Eq. 18). The explanation for the $\mathrm{EBG_c}$ follows Eq. 16. Thus, the adjusted equation to estimate the net protein requirement is presented:

$$\mathrm{NP_g} = 1.0348 \times 0.6919 \times \left(\mathrm{EBW_{open}^{-0.3081}}\right) \times \mathrm{EBG_c}$$

$$(R^2 = 0.901,\ \mathrm{RMSE} = 3.526), \quad [16]$$

where $\mathrm{NP_g}$ is measured in grams per day, $\mathrm{EBW_{open}}$ is measured in kilograms, and $\mathrm{EBG_c}$ is measured in kilograms per day.

The EBG was adjusted to represent only body gain due to carcass and organ gain (discounting GEST growth due to gestation). Thus, using pregnant animals, the proportion of GEST due to pregnancy was calculated in the EBG, according to Eq. 17 (Figure 6):

$$\text{Proportion of GEST} = 0.004497_{\pm 0.005489} \times \exp^{0.01198_{\pm 0.004097} \times \mathrm{GD}}$$

$$(R^2 = 0.731,\ \mathrm{RMSE} = 0.027), \quad [17]$$

where "Proportion of GEST" is the proportion of gestation components due to pregnancy in the EBG. The $\mathrm{EBG_c}$ was then calculated as

$$\mathrm{EBG_c} = \mathrm{EBG} \times (1 - \text{proportion of GEST}), \quad [18]$$

where EBG is measured in kilograms per day, and GEST is the sum of all gestation components (%). For all requirements for gain estimated subsequently, $\mathrm{EBG_c}$ was used in place of EBG.

As expected, BCP and $\mathrm{NP_g}$ had inverse relationships with EBW. The BCP increases due to animal growth, and more protein is deposited. However, $\mathrm{NP_g}$ decreases with greater BW due to variation in gain composition. Comparing the $\mathrm{NP_g}$ of an adult Zebu cow with a growing heifer (350 kg), the estimated $\mathrm{NP_g}$ of a cow with BCS 3.5 is about 40% lower than the estimated $\mathrm{NP_g}$ for the growing heifer. The Brazilian system for beef cattle (BR-CORTE; Valadares Filho et al., 2016) reported that this difference is due to variations in gain composition, because growing heifers have a more significant proportion of lean tissue gain than adult cows in average BCS.

Using an equation proposed by the NRC (2001) and considering a cow that weighs 450 kg with ADG of 0.3 kg/d, the estimated $\mathrm{NP_g}$ would be 43 g/d. However, the present study suggests an $\mathrm{NP_g}$ of 33.5 g/d for the same animal, which is a 22% difference in $\mathrm{NP_g}$ requirements. The crossbred cows evaluated in this study had a BW close to maturity at the beginning of the experiment, approximately 450 kg. In this context, considering a

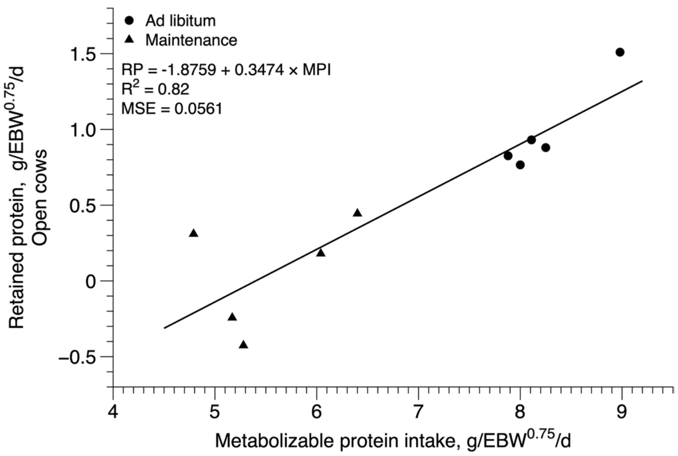

Figure 7. Retained protein (RP, g $EBW^{0.75}$/d) of pregnant Holstein × Gyr dairy cows fed ad libitum or maintenance diets, according to metabolizable protein intake (MPI, g $EBW^{0.75}$/d). EBW = empty BW; MSE = mean squared error.

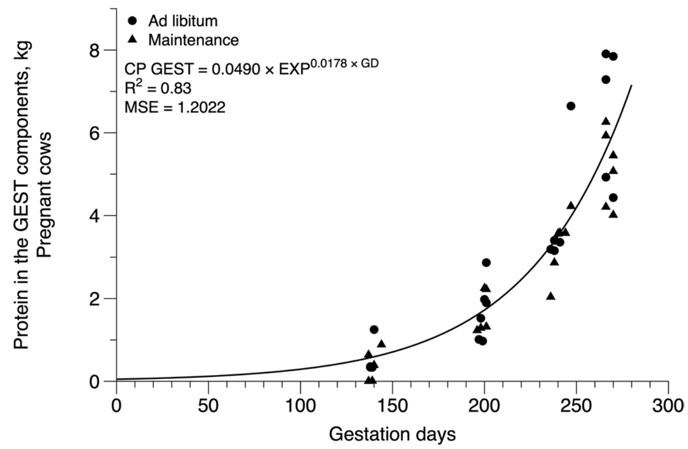

Figure 8. Total CP (kg) in gestational components (GEST: uterus + placenta + fetus + fetal fluids + mammary gland) of pregnant Holstein × Gyr dairy cows fed ad libitum or maintenance diets, according to gestation days. MSE = mean squared error.

cow fed a diet balanced adequately for energy, the proportion of body protein decreases and the proportion of body fat increases with an increase in EBW (NASEM, 2016).

The efficiency of protein use for gain (k), estimated as the slope of the regression between the RP (g $EBW^{0.75}$/d) in the animal's body and the MPI (g $EBW^{0.75}$/d, Figure 7), was calculated as follows:

$$\mathrm{RP} = -1.8759_{\pm 0.4088} + 0.3474_{\pm 0.05807} \times \mathrm{MPI}$$

$$(\mathrm{R}^2 = 0.820,\ \mathrm{RMSE} = 0.237). \quad [19]$$

The estimated k in this study was 34.7%. The NRC (2001) suggests the equation proposed by Ainslie et al. (1993) to calculate the k of animals with a BW of less than 478 kg and 28.9% for animals with a BW greater than 478 kg. Silva et al. (2018) suggested a k value of 25% for Holstein × Gyr dairy heifers with an average BW of 251 kg. In a study evaluating tropical cattle, Oss et al. (2017) reported a k value of 35.7% for growing Holstein × Gyr bulls. Although there are differences (i.e., sex and BW) between cows used by previous authors and the current study, some reported k values were close to ours (Ainslie et al., 1993; Oss et al., 2017). We speculate that the breed, and thus the gain composition of the animals, may explain the differences found herein; however, no studies were designed to answer specific questions, and only speculation is possible. Additionally, Garrett (1980) reported that the variation in efficiency estimates for protein and fat synthesis might be related to the differences in the diet composition and, therefore, in the end, products of digestion.

Requirements for Gestation

The requirements for gestation were calculated based on protein accumulation in GEST, which is the difference between the gestation components (udder, uterus, and all the other components of the gravid uterus) observed in the pregnant cow and the gestation components of that same cow if it were a nonpregnant animal (Figure 8). We used a simple exponential model to adjust the net protein requirement for gestation as a function of GD. In the current study, the approach adopted to calculate the gestation requirements was first described by Sguizzato et al. (2020a):

$$\mathrm{CP\ GEST} = 0.0490_{\pm 0.02701} \times \exp^{0.01784_{\pm 0.00207} \times \mathrm{GD}}$$

$$(\mathrm{R}^2 = 0.830,\ \mathrm{RMSE} = 1.096), \quad [20]$$

where CP GEST is the protein in GEST due to gestation exclusively (kg).

The first derivative of Eq. 20 added a correction factor for expected calf BW (35 kg), considered as the net protein requirement for gestation (NP_{gest}):

$$\mathrm{NP_{gest}} = 0.0008722 \times \exp^{(0.01784 \times \mathrm{GD})} \times (\text{calf weight}/35)$$

$$(\mathrm{R}^2 = 0.820,\ \mathrm{RMSE} = 0.237), \quad [21]$$

where NP_{gest} is measured in kilograms per day and includes the protein retained in the gravid uterus and mammary gland.

The updated version of NASEM (2021) reported the average birth weight for many breeds, such as Holstein

(44), Jersey (26), Ayrshire (38), Brown Swiss (48), and Guernsey (36); however, there is no information for crossbred calves. In the current study, calf BW (35 kg) was obtained according to the average birth weight of Holstein × Gyr calves from Silva et al. (2017).

Another important point to consider is the precise moment when pregnancy requirements should be added to dietary requirements. After estimating protein requirements for pregnancy, we determined the day of gestation when pregnancy requirements were statistically different from nonpregnant cows using the lower confidence limit of the protein in GEST (Figure 8, $P < 0.05$). Our data indicated that protein requirements for pregnancy should be accounted for from 140 d onward (Figure 8). The NRC (2001) suggests that pregnancy requirements need to be accounted for starting at d 190 of pregnancy; INRA (2018) suggests they should be accounted for only during the last third of gestation; and NASEM (2021) recommended starting to compute the requirements at 12 d in gestation. The data show that the last third of gestation might be too late to start adding pregnancy requirements, but the requirements are probably minimal at the beginning of gestation (≈1.6 g of metabolizable protein with 10 GD) and within the margin of error of any diet formulation. Therefore, 140 d in gestation seems a reasonable number.

The efficiency of use of metabolizable protein for gestation (k_{gest}) was calculated by the iterative method (Eq. 12). The deviation between estimated MPI and MP_m was zero when $k_{gest} = 62.5\%$. This value is close to that suggested by NASEM (2016), of 65% for beef cattle. However, the eighth revised version of NASEM (2021) for dairy cattle suggests a 33% protein efficiency value during gestation. The committee reported that available data were insufficient to change the protein efficiency estimated in the previous edition of NRC (2001). However, our data, aligned with those of NASEM (2016) and BR-CORTE (Valadares Filho et al., 2016) for beef animals, suggest a k_{gest} greater than 33%. Although we understand that some breed differences might be observed between Holstein × Gyr and Nellore (in the case of BR-CORTE, Valadares Filho et al., 2016) and Angus (in the case of NASEM, 2016), it is not likely so large (from 62.5 to 33%). Additionally, using the data obtained for our animals (Rotta et al., 2015c), we estimated the efficiency of truly digestible protein according to INRA (2018) as 75.8%. Therefore, based on the most recent data, we suspect that k_{gest} is most likely closer to 62.5% than to 33%. It is important also to highlight that k_{gest} will probably change with the MPI AA profile. However, as we had only one diet, no significant inferences were made in this study, but it could partially explain some of those differences between systems.

It is essential to point out the lack of data for nutrient accretion in the conceptus tissues as fetus, placenta, fetal fluids, and uterus as gestation advances (NASEM, 2021). Although the revised edition calculated the size of the gravid uterus based on the calf birth weight, the rate of involution of the uterine tissue and the fate of the AA of the involuting tissue are unknown (NASEM, 2021). Furthermore, given the intensive selection for milk production over the past 50 years, research has suggested that modern dairy cows have greater metabolic rates than before (NASEM, 2021). For example, NASEM (2021) reports that estimated MP requirements for gestation by a dry cow producing a calf with a birth weight of 44 kg is about 37% greater at 5 d prepartum compared with NRC (2001). Overall, in the current study, from 140 to 275 GD, our estimates of MP_{gest} were 30% lower than those described by NASEM (2021), whereas the INRA (2018) estimation of MP_{gest} of crossbred cows was about 36% lower (Figure 9). Based on our results, we can speculate a second hypothesis. While acknowledging the increased productivity of modern dairy cows, an opportunity exists to enhance their efficiency rather than simply increasing their requirements, as suggested by our k_{gest}. Another crucial factor to take into account is the variability in MPI estimates. Therefore, it is essential to acknowledge that different methods of estimating MPI are likely to produce diverse estimates, ultimately affecting k_{gest}, and consequently influencing our estimated MP requirements. Hence, further studies are imperative to clarify and reconcile these disparities. Additionally, we used only one diet in this study, and the imprecision in MPI estimates from other diets should also be further investigated.

Furthermore, the current study's results align with those of a previous study from our laboratory, which reported lower net energy requirements for gestation (NE_{gest}) in crossbred cows until 230 GD (Sguizzato et al., 2020a). However, after this period, the estimated net energy requirements for pregnancy (NE_{preg}) were greater than NRC (2001) predicted. The previous authors cited suggested a greater increase of retained energy in the gravid uterus than in the mammary gland, to support the fetus's development during the final gestation period. In addition, mammary gland changes account for requirements for pregnancy in a smaller proportion than uterus involution (Sguizzato et al., 2020a). Therefore, according to the previous author, the balance of the pregnancy components needs to be considered when evaluating gestation requirements. Other authors reported a greater demand for glucose and AA by the placenta during pregnancy (McNeill et al., 1997; Freetly and Ferrell, 1998, 2000). As pointed out earlier, data about AA metabolism for gestating

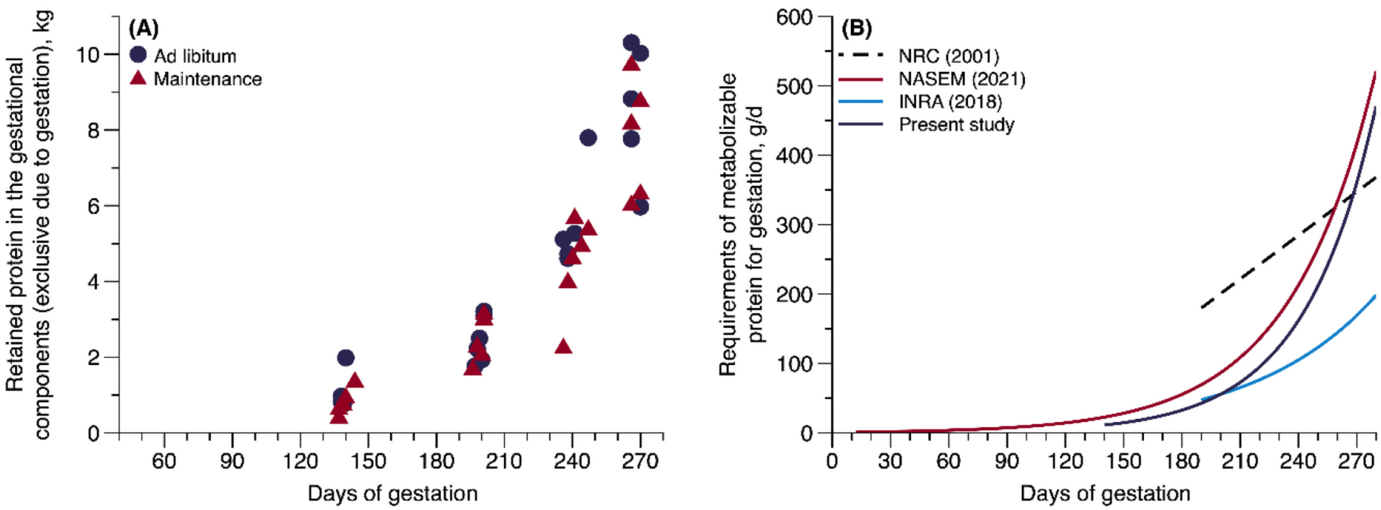

Figure 9. Exponential relationship between days of gestation and crude protein retained in the gestational components (uterus + placenta + fetus + fetal fluids + mammary gland; A); and prediction of the requirements of metabolizable protein for gestation proposed by NRC (2001), NASEM (2021), and INRA (2018) compared with the model proposed by the present study in days of gestation (B). Calculations were made considering a calf birth weight of 35 kg.

and nonlactating cows are lacking, and it is estimated that for female ruminants fed 110 to 140% of their protein requirement in late pregnancy, approximately 80% of the digested protein passes through the gravid uterus (Bell and Ehrhardt, 2000).

To summarize the results, we calculated the total metabolizable requirements for a cow with 450 kg BW, 0.3 kg/d EBG, BCS 3.5, expected calf weight of 35 kg, and 240 GD, using all international systems and the current model. According to our data, we estimated the total MP requirements as 536 g/d. According to NRC (2001) these requirements are 730 g/d; according to NASEM (2021), the requirements are 573 g/d; and according to INRA (2018), the requirements are 709 g/d (requirements of protein digestible in the small intestine, PDI). It is noticeable that the most recent recommendations (the present study and NASEM, 2021) lean toward lower requirements during gestation. Nutrient requirement calculations depend on how much we feed the animals. As the current trend is to feed less protein daily, with a better AA balance and greater efficiency, the result is a recommendation of less MP. This is represented by the results found herein; nevertheless, NASEM (2021) used the same database as NRC (2001) but with a different statistical approach; thus, this justification does not fit into their situation. It is crucial to emphasize the need to avoid mixing different methods for estimating microbial protein requirements. Specifically, if the current method is employed to estimate MP requirements for pregnant cows, it is advised not to use NASEM or other estimation approaches. Furthermore, the proposed method relies on the estimation of microbial protein, which, in our case, utilizes urinary estimates. This requirement represents a significant limitation for the practical application of the proposed approach.

CONCLUSIONS

The proposed equations to estimate the protein requirements during the gestation were different from those reported by other systems such as INRA (2018), NRC (2001), and NASEM (2021). We suggest an exponential model to describe protein requirements for pregnancy for dairy cows. However, our study was conducted with 1 diet fed at 2 intake levels. Although we used nonlactating cows and a diet with low CP level (thus, likely a low passage rate and greater proportion of RDP), inferences to lactating animals and high-protein diets should be made with care.

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