Effect of experimental design on responses to 2 concentrations of metabolizable protein in multiparous dairy cows

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ABSTRACT

The objective of this research was to characterize the implications of changing between diets formulated to be adequate (ADMP) or low (LOMP) in metabolizable protein in a Latin square (LSq) design or of feeding the same diets continuously in a randomized complete block experimental design (RCBD). Fifty-four multiparous early-lactation cows (initial average $\pm$ SD; parity 2.8 ± 0.9 , 85.8 ± 31 d in milk, 715 ± 63 kg of body weight, 29.1 ± 2.7 kg of dry matter intake/d, and 57.7 ± 5.7 kg of milk yield/d) were blocked by parity and days in milk and were then randomly assigned to experimental design, with 16 cows assigned to LSq and 38 cows assigned to RCBD. Cows within blocks in LSq were randomly assigned to sequence in a 4-sequence, 4-period, 2-treatment LSq balanced for the effects of previous treatment carryover. Cows within blocks in RCBD were randomly assigned to dietary treatment, which was fed over the same four 28-d periods as the cows in LSq. Treatment diets were formulated to be similar in composition with the exception of exchanging an equal quantity of expeller soybean meal from ADMP (16.5% crude protein; 28.4% ash-free, amylase-treated neutral detergent fiber organic matter) for soybean hulls in LOMP (14.6% crude protein; 31.1% ash-free, amylase-treated neutral detergent fiber organic matter). Cows were individually fed treatment diets in a tiestall barn once daily for ad libitum consumption, milked 3 times daily, and administered recombinant bovine somatotropin every 14 d. Milk yield and feed offered and refused were measured daily; BW was recorded on 2 consecutive days each week; milk composition was measured at 6 consecutive milkings each week; and spot samples of feces, urine, and blood were collected during the last week of each period and a covariate period. Experimental designs were analyzed separately using results from wk 4 of each period with mixed effects modeling. Dry matter intake and milk fat yield were not affected by

diet in either design, whereas milk and protein yields were greater for cows fed ADMP in both designs. Milk fat and protein percentage responses and milk energy output inferences were different between designs. Milk fat yield and percentage responses were affected by previous treatment carryover in LSq. Metabolic and digestibility inferences were very similar between designs. Under the conditions of this experiment, inferences on N metabolism and the majority of production measurements were not affected by experimental design, with the principal exceptions of milk fat and protein percentage and milk energy output.

Key words: metabolizable protein, milk production, experimental design

INTRODUCTION

Providing optimum levels of dietary protein is important to enhance productivity while increasing N efficiency and reducing N excretion to the environment (Huhtanen and Hristov, 2009; Powell et al., 2010). To enable dairy farmers and their consultants to efficiently and cost effectively meet these nutritional requirements of the dairy cow, recommendations are made based on the results of designed experiments. For the inferences from these experiments to be valid in an on-farm setting, the conditions of the experiment should represent the conditions in which the data will be used. Frequently, nutritional experiments are conducted in such a way that cows receive several treatment diets for short periods of time compared with what might be expected in practice. In particular, these shorter durations are used when an animal may change between dietary treatments as part of a changeover experimental design such as a switchback or Latin square (Lucas, 1960). The extent to which these design considerations affect the inferences of experiments has been of interest for some time (Cochran et al., 1941) and has been the subject of recent research relevant to protein and AA feeding (Benefield et al., 2009; Lee et al., 2015) or analyzed using literature data in meta-analyses (Huhtanen and Hetta, 2012; Zanton, 2016). Two principal questions are evident when using short-term experiments to estimate

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responses that would be applied to long-term feeding situations: is enough time allowed so that the animal can adequately adapt to the new treatment, and does the nutritional history of the animal affect the results (Ferrell et al., 1986) because carry-over effects of previous treatments are different? This has been especially emphasized with respect to dietary protein because of the possibility of changing the dynamics of body protein degradation and synthesis by relying on body protein reserves (Paquay et al., 1972) and particularly for some AA, which have sizable storage pools that need to reach equilibrium to the new AA status (e.g., histidine; Giallongo et al., 2016). However, recent meta-analyses of responses in lactating dairy cow nutrition experiments have found that changeover studies and continuous studies result in similar inferences except when treatments are extreme, in which case the changeover design underestimated the response observed in the continuous study (Huhtanen and Hetta, 2012; Zanton, 2016). Literature analyses are very valuable to address the implications of experimental design across a wide range of dietary conditions; however, design-specific differences in animal or dietary characteristics and frequently experimental objectives and sampling intensity were evident in both meta-analyses. Furthermore, no direct comparison of continuous and changeover designs in lactating dairy cows was available that was not otherwise confounded by differences in objective or sampling intensity (Zanton, 2016). Therefore, the objective of this study was to evaluate the effects of 2 levels of MP on lactation performance, digestibility, and metabolism when dairy cows are fed diets continuously or according to a changeover experimental design. The hypothesis was that inference about treatment effects would be independent of experimental design (changeover or continuous) under conditions in which MP concentration was predicted to be either adequate or low.

MATERIALS AND METHODS

Cows, Experimental Designs, and Diets

All experimental procedures involving the use of animals were approved by the University of Wisconsin Institutional Animal Care and Use Committee (protocol no. A005043). To meet the objective of this research, 54 early-lactation multiparous Holstein cows were selected from the research herd at the Dairy Forage Research Center dairy farm in Prairie du Sac, Wisconsin. Cows were in lactation 2 to 5 and were 43 to 142 DIM at the beginning of the covariate period; summary descriptions of the cows collected before the initiation of the study during the covariate period are reported in Table 1. The study consisted of a 14-d covariate period followed

by a 112-d experimental period during which cows were fed their treatment diets either continuously according to a randomized complete block design (**RCBD**) or according to a replicated 4-period, 4-sequence, 2-treatment Latin square design (**LSq**) with 28-d periods that was balanced for carry-over of previous treatment (design 4.4.13 in Jones and Kenward, 2003). Cows were balanced according to lactation number and blocked by DIM ($n = 4$: starting DIM <60, 61–100, 101–120, and >121) and then randomly allocated to RCBD ($n = 38$) or LSq ($n = 16$). Cows in either design were randomly distributed throughout the barn, and management and experimental procedures were conducted identically irrespective of design, with the only difference being that cows in LSq could change diets between periods.

Cows were individually fed once daily and housed in cross-ventilated tiestalls during the 4-mo study with feed provided and water available for ad libitum consumption and recombinant bovine somatotropin (500 mg of Posilac, Elanco Animal Health, Greenfield, IN) administration every 14 d (d 12 and 26 of each 28-d period). Feed was offered between approximately 0700 and 0800 h at between 105 and 110% of expected consumption, and feed refusals were collected at approximately 0500 h. Cows were milked 3 times daily at approximately 0400, 1100, and 1900 h. For 14 d before the initiation of treatment diet feeding, cows were continued on the common herd diet, after which time the treatment diet feeding commenced (Table 2). Treatment diets were formulated to promote differences in primary production measurements such as milk production and to have these differences be as extreme as possible within the context of changing dietary protein at levels considered commercially practical (Hristov et al., 2018). To this end, diets were formulated that were predicted to be adequate in MP (**ADMP**; based on NRC, 2001) or to be low in MP (**LOMP**) by exchanging expellers soybean meal for soybean hulls, resulting in diets that were 16.5 and 14.6% CP, respectively (Table 2). As-fed composition was adjusted weekly based on DM measurements to maintain dietary ingredient and CP concentration (Supplemental Figure S1, <https://doi.org/10.3168/jds.2018-15730>).

Sampling and Laboratory Analysis

Temperature and humidity measurements were taken every 15 min throughout the study at 2 locations in the barn using an EL-USB-2-LCD data logger (Lascar Electronics Inc., Erie, PA) and temperature-humidity index was calculated (NRC, 1971). Samples of forages, TMR, high-moisture corn, and orts were collected daily, composited weekly, and stored at -20°C until analysis. Samples of dried distiller grains with solubles, roasted

Table 1. Covariate period characteristics of cows assigned to the Latin square design (LSq; $n = 14$) or randomized complete block design (RCBD; $n = 32$)¹

Item	LSq		RCBD	
	Average	SD	Average	SD
Lactation no.	2.6	0.93	2.9	0.94
Age, mo	46.9	12.08	51.2	12.33
DIM	89.7	33.38	84.1	30.10
BW, kg	704	73	720	59
DMI, kg/d	29.0	3.02	29.1	2.58
Milk, kg/d	56.6	3.67	58.1	6.39
Protein, kg/d	1.56	0.126	1.56	0.141
Protein, %	2.75	0.149	2.70	0.183
Fat, kg/d	2.17	0.297	2.21	0.263
Fat, %	3.83	0.359	3.81	0.384
Lactose, kg/d	2.84	0.203	2.86	0.307
Lactose, %	5.02	0.134	4.93	0.161
MUN, g	7.08	0.736	7.11	1.193
MUN, mg/dL	12.54	1.183	12.24	1.827
NE _L , Mcal	40.2	3.90	40.6	3.95
NE _L /DMI, Mcal/kg	1.4	0.12	1.4	0.16
Milk protein N/N intake, g/100 g	30.8	1.90	30.9	2.68

¹Measurements were taken before treatment diet feeding on cows that completed the experiment.

soybeans, canola meal, soybean hulls, and expeller soybean meal were collected once weekly and stored at room temperature. Feed, TMR, and ort samples were thawed at room temperature, dried at 55°C for approximately 72 h, and ground through a 1-mm screen (Wiley mill, Arthur H. Thomas Co., Philadelphia, PA). Weekly feed samples were analyzed for DM, NDF, and starch by Dairyland Laboratories Inc. (Arcadia, WI) to monitor compositional analysis (Supplemental Figure S1; <https://doi.org/10.3168/jds.2018-15730>). Feed, TMR, and orts for the sampling weeks were analyzed for DM (method 934.01; AOAC, 1990), ash (method 938.08; AOAC, 1990), N (Leco N Analyzer, Leco Corp., St. Joseph, MI), and NDF determined with α -amylase, Na₂SO₃, exclusive of ash (**aNDF_{OM}**) including thermo-stable α -amylase and Na₂SO₃ using the Ankom200 Fiber Analyzer (Ankom Technology, Macedon, NY) reported exclusive of residual ash by ashing neutral detergent residue and filter bags after extracting neutral detergent solubles. Indigestible **aNDF_{OM}** was determined in feedstuffs, TMR, and orts by incubating F57 filter bags (25-mm porosity, 5 × 5 cm², 350-mg sample; Ankom Technology) for 12 d in the rumen of 2 cows and analyzing residual sample for **aNDF_{OM}** using the methodology previously described (Lee et al., 2012b). Dietary chemical composition and intake were calculated based on the chemical analysis of the individual ingredients and their contribution to the DM of the TMR where ort composition was assumed to be the same as the TMR offered to mitigate against nonrepresentative sampling. Predictions (NRC, 2001) of NE_L, MP, RDP, and RUP were made using feed analyses augmented with tabular values based on a common level of production observed

during the covariate period, which would not include changes in DMI and production that would be caused by treatment diet and would therefore represent an estimation of the formulated diet and not an estimation of the consumed diet (Table 2).

Milk yields were recorded at each milking throughout the study. Milk samples for composition were collected at 6 consecutive milkings at 7-d intervals (d 3–4, 10–11, 17–18, and 24–25 of each consecutive 28-d period for both designs) throughout the entire study and analyzed for fat, protein, lactose, and MUN by near-infrared spectroscopy (AgSource, Verona, WI; Foss FT6000 instrument, Foss Electric, Hillerød, Denmark; method 972.16; AOAC, 1990). Milk component yield was calculated using the concentration of milk components at each milking multiplied by the milk yield at each milking and summed over the sampling day. Milk protein N was calculated from milk true protein output as milk true protein/6.38, and milk N output was calculated as the sum of MUN and milk protein N output. Milk NE_L output was calculated using the formula reported by NRC (2001). On the same days as milk sample collection each week, cows were weighed after the 1900 h milking.

On d 24 to 25 of each period for both designs, spot samples of urine, feces, and blood were collected beginning at 1200 h (d 24) and 0000 h (d 25; beginning approximately 4 to 5 h and 16 to 17 h after d 24 feeding, respectively). Urine was collected after voluntary urination by the cow or upon mechanical stimulation of the vulva. Urine samples were immediately acidified by diluting 15 mL of urine with 60 mL of 0.072 *N* H₂SO₄ and stored at –20°C until analysis. Urine was thawed

Table 2. Ingredient and chemical composition for adequate-MP (ADMP) and low-MP (LOMP) diets fed to early-lactation dairy cows according to a Latin square design or a randomized complete block design

Item, % of DM unless noted	Covariate	ADMP	LOMP
Corn silage	25.7	37.8	37.6
Alfalfa silage	24.6	15.9	15.8
Ground high-moisture corn	26.5	19.7	19.8
Liquid molasses blend ¹	—	2.5	2.5
Dried distillers grains plus solubles	4.1	4.0	4.0
Roasted soybeans	7.8	5.0	5.0
Canola meal	8.7	5.0	5.0
Soybean hulls	—	2.0	7.8
Expeller soybean meal ²	—	5.6	0.0
Vitamins and minerals ³	2.5	2.5	2.5
CP	17.8	16.5	14.6
aNDF _{OM} ⁴	28.0	28.4	31.1
pdNDF _{OM} ⁴	20.5	20.9	23.6
iNDF _{OM} ⁴	7.5	7.5	7.5
Forage NDF _{OM} ⁴	17.5	20.6	20.5
NE _L , ⁵ Mcal/kg	1.59	1.57	1.54
MP ⁵	10.4	11.1	9.5
RDP ⁵	12.2	10.1	9.6
RUP ⁵	5.8	6.5	5.0
NE _L balance, ⁵ Mcal/d	−5.6	−6.3	−7.2
MP balance, ⁵ g/d	−319	−107	−573
RDP balance, ⁵ g/d	668	64	−44

¹Formulated to contain (as-fed basis) 73% cane molasses, 17% water, and 10% glycerin. Provided by Westway Feed Products (Tomball, TX).

²SoyPlus provided by Landus Cooperative (Ames, IA).

³Vitamin and mineral mix contained Ca, 16.7% of DM; Na, 15.8% of DM; Cl, 7.1% of DM; Mg, 4.6% of DM; K, 0.6% of DM; S, 1.0% of DM; Co, 45 mg/kg; Cu, 554 mg/kg; I, 64 mg/kg; Fe, 830 mg/kg; Mn, 2,776 mg/kg; Se, 16 mg/kg; Zn, 2,997 mg/kg; monensin, 522 mg/kg; vitamin A, 309 kIU/kg; vitamin D, 62 kIU/kg; vitamin E, 1,454 IU/kg.

⁴aNDF_{OM} = neutral detergent fiber determined with α-amylase, Na₂SO₃, exclusive of ash. pdNDF_{OM} = potentially digestible aNDF_{OM} (difference between aNDF_{OM} and iNDF_{OM}). iNDF_{OM} = indigestible aNDF_{OM} based on 12 d in the rumen.

⁵Predicted from NRC (2001) based on ingredient chemical composition, tabular values, and cow production values recorded during the covariate period.

at room temperature and analyzed for total N using an N analyzer (TruMac N, Leco Corp.), urea using a colorimetric method (Broderick and Clayton, 1997), and creatinine using a picric acid method (Oser, 1965) adapted to flow injection analysis (QuickChem 8500, Lachat Instruments, Loveland, CO). Urine output was estimated by assuming that 29 mg of creatinine/kg of BW is excreted (Valadares et al., 1999), although the use of this equation may underestimate the variance of urine output and subsequent nutrient excretion based upon it (Tebbe and Weiss, 2018). Output of urinary N and urea was calculated by multiplying the estimated urine output by the concentration of the analyte in the urine.

Fecal samples were collected as the cows defecated or were collected manually from the rectum. These

samples were then immediately dried at 55°C for approximately 72 h, composited on an equal DM basis, and ground through a 1-mm screen (Wiley mill, Arthur H. Thomas Co.). Ground samples were subsequently analyzed using the procedures previously described for feed. Fecal output was estimated using indigestible aNDF_{OM} as an internal marker:

fecal DM =

$$\frac{\text{iNDF}_{\text{OM}} \text{ intake (g/d)}}{\text{fecal iNDF}_{\text{OM}} \text{ concentration (g of iNDF}_{\text{OM}}/\text{kg of DM)}}$$

Blood samples were collected in tubes with K₃-EDTA as an anticoagulant (Vacutette, Greiner Bio-One North America, Monroe, NC) by venipuncture of the coccygeal artery or vein after the completion of the feces and urine collection. Samples were stored on ice until plasma was separated (1,500 × *g*, 4°C, 15 min), aspirated to aliquots, and stored at −20°C until analysis. Individual aliquots were analyzed for glucose (Auto-kit Glucose, Wako Diagnostics, Mountain View, CA), plasma urea N (PUN; QuantiChrom Urea Assay Kit, BioAssay Systems, Hayward, CA), nonesterified fatty acids (NEFA; NEFA-HR(2), Wako Diagnostics), and insulin (bovine insulin ELISA, Mercodia AB, Uppsala, Sweden) using commercial kits. Before analyzing for PUN, plasma was deproteinized by combining 0.5 mL of plasma with 0.5 mL of 5% trichloroacetic acid, which was immediately vortexed. This combination sat at room temperature for 10 min, and the supernatant was harvested after 10 min of centrifuging at 12,000 × *g* at room temperature.

Statistical Analysis

For consistency between designs and among production, digestibility, and metabolic responses, statistical analysis for treatment effect least squares means was conducted on data for the last week of each period for both designs. The model for the data in LSq was

$$Y_{ijklm} = \mu + Cov_i + S_j + T_{k;j} + R_l + P_{m;jk} + c_{i;j} + e_{ijklm}$$

where Y_{ijklm} = the dependent variable; μ = the overall mean; Cov_i = the covariate measurement for cow *i*; S_j = the fixed effect of square *j*, where *j* = 1 to 4 squares (same as DIM blocks, below); $T_{k;j}$ = the fixed effect of treatment *k* within square *j*, where *k* = 1, 2; R_l = the fixed effect of previous treatment *l*, where *l* = 1, 2; $P_{m;jk}$ = the fixed effect of period *m* within square *j* and treat-

ment k , where $m = 1$ to 4; c_{ij} = the random effect of cow i within square $j \sim N(0, \sigma_c^2)$; and e_{ijklm} = the random residual $\sim N(0, \sigma_e^2)$, where σ_c^2 and σ_e^2 are the error variances associated with cow and residual effects, respectively.

The statistical model for analysis of RCBD was

$$Y_{ijkl} = \mu + Cov_i + B_j + T_{kj} + W_{l;jk} + c_{i;jk} + e_{ijkl},$$

where Y_{ijkl} = the dependent variable; μ = the overall mean; Cov_i = the covariate measurement for cow i ; B_j = the fixed effect of block j , where $j = 1$ to 4; T_{kj} = the fixed effect of treatment k within block j , where $k = 1, 2$; $W_{l;jk}$ = the fixed effect of week l within block j and treatment k , where $l = 4, 8, 12$, or 16 wk; $c_{i;jk}$ = the random effect of subject i within block j and treatment $k \sim N(0, \sigma_c^2)$; and e_{ijkl} = the random residual $\sim N(0, \sigma_e^2)$.

Statistical analysis was conducted using the mixed procedure of SAS (SAS Institute, 2013). For comparison between designs, for least squares means calculation, the value for Cov_i was based on the overall average response measured during the covariate period and not based on the average for each design. Treatment and treatment $\times$ week interaction effects and tests were calculated from the above reduced models within PROC MIXED. Denominator degrees of freedom and small-sample corrections to the standard errors were conducted using the Kenward-Roger adjustment. Auto-correlation between repeated measures in the RCBD was modeled using the first-order auto-regressive covariance structure. To compare the evidence resulting from the different designs, t -scores of the treatment differences were calculated. From these, a concordance correlation coefficient (Lin, 1989) was calculated using the epiR package in R (R Core Team, 2015). Differences were considered significant when $P < 0.05$.

RESULTS AND DISCUSSION

General Considerations

The cows enrolled in this study were all multiparous and in early to mid lactation. Throughout the course of the experiment, 8 cows had to be removed for various reasons that did not appear to be related to dietary treatment or experimental design, and the responses for these cows were not used in any analysis. The remaining 46 cows averaged ($\pm$ SD) 29 ± 2.69 kg of DMI/d and 57.7 ± 5.7 kg of milk yield/d and weighed 715 ± 63 kg (Table 1). Cows within blocks were randomly assigned to experimental design, and the observed covariate responses were similar between designs. No

differences were found in covariate characteristics, so for ease of comparing the responses between the designs, least squares means were covariate adjusted to the common value observed across all cows during the covariate period.

To achieve the objective of this study, diets were designed to promote treatment differences by changing both CP concentration and degradability to enable the responses in the different designs to be studied as robustly as possible. Within this context, dietary MP was varied by exchanging expeller soybean meal for soybean hulls with all other ingredients formulated to be the same between diets (Table 2). Changing ingredients with these different compositions (Table 3) resulted in different CP and NDF concentrations between diets as expected. Based on pretrial information, the formulation of ADMP was to be adequate in MP; however, using the actual measured parameters, NRC (2001) predicted that this diet resulted in a negative MP balance. Lee et al. (2012b) reported that NRC (2001) underpredicted milk yield in diets that were estimated to be MP deficient, which was observed in this experiment only for LOMP and not ADMP. Cows fed ADMP yielded 55.7 kg of milk/d with an NRC prediction of 55.0 kg/d of MP allowable milk, whereas LOMP cows yielded 53.0 kg of milk/d with an NRC prediction of 43.5 kg/d of MP allowable milk. These observations are consistent with the biological concept of a variable efficiency of MP use for productive purposes (Metcalf et al., 2008; Moraes et al., 2018) compared with the constant efficiency used by the NRC (2001) model. Although the magnitude of the NRC-predicted production differences between diets was not achieved, cows were observed to be sensitive to these levels of dietary MP changes. Therefore, the inferences resulting from the different experimental designs were evaluated when observed treatment responses were significant but not extreme.

At the beginning of any nutritional experiment, including RCBD, the cows' diets need to be changed to meet the objectives of the study. Frequently, whether part of a changeover or a continuous experimental design, an adaptation period is provided to allow the cow to respond to the treatment diet, although the amount of time that is optimal for this to occur is not completely known for most situations. In the literature data used for a previous meta-analysis (Zanton, 2016), cows in changeover designs were adapted to the diet for an average of 13 d, whereas cows in continuous experiments were adapted for an average of 3 d before data or sample collection began. In this experiment, cows for both designs were fed the treatment diets for a minimum of 24 d before sample collection began

Table 3. Ingredient chemical composition (% of DM unless noted; SD in parentheses)

Item	DM, ¹ % as fed	Ash ²	CP ¹	aNDF _{OM} ^{1,3}	pdNDF _{OM} ^{2,3}	iNDF _{OM} ^{2,3}	Starch ¹
Corn silage	35.74 (1.63)	4.12 (0.62)	7.65 (0.42)	39.57 (2.36)	30.00 (3.29)	10.13 (1.74)	33.82 (2.30)
Alfalfa silage	43.39 (6.08)	11.47 (0.70)	23.84 (0.91)	37.51 (2.34)	17.67 (1.21)	16.08 (1.68)	—
Ground high-moisture corn	71.31 (2.51)	1.96 (0.08)	8.66 (0.39)	8.65 (1.91)	6.97 (1.77)	0.56 (0.65)	70.58 (1.18)
Dried distillers grains plus solubles	88.52 (0.89)	5.69 (0.15)	30.06 (1.57)	29.75 (3.15)	31.97 (1.31)	2.45 (1.44)	—
Roasted soybeans ⁴	96.17 (0.38)	5.63 (0.14)	37.83 (1.62)	19.37 (6.24)	25.49 (2.07)	3.19 (2.07)	—
Canola meal	90.37 (0.71)	8.78 (0.61)	40.44 (1.07)	26.67 (2.53)	14.78 (1.25)	11.54 (1.31)	—
Soybean hulls	90.38 (0.69)	5.65 (0.23)	12.23 (0.57)	65.30 (1.77)	61.19 (0.68)	2.45 (0.54)	—
Expeller soybean meal	90.79 (0.69)	6.77 (0.12)	47.20 (0.93)	14.01 (1.06)	13.96 (1.77)	0.64 (0.77)	—

¹Based on weekly samples.²Based on samples collected during the weeks of sampling.³aNDF_{OM} = neutral detergent fiber determined with α -amylase, Na₂SO₃, exclusive of ash. pdNDF_{OM} = potentially digestible aNDF_{OM} (difference between aNDF_{OM} and iNDF_{OM}). iNDF_{OM} = indigestible aNDF_{OM} based on 12 d in the rumen.⁴Roasted to a protein dispersibility index of 9 to 11 (Wu and Satter, 2000).

for analyzing the responses due to dietary differences. Weekly production responses are shown graphically for illustrating time-course responses to treatments.

Production Responses

Body weight and BW change were not affected by treatment diets (Table 4). The diets in this study generally resulted in DMI that was initially higher than that observed during the covariate period and declined throughout the study, although the contributions of DIM cannot be separated from the experimental periods (Figure 1). This treatment $\times$ time interaction in DMI is an important response that could have practical relevance on dairy farms and for the interpretation of experiments. However, this type of response is not observable in experiments designed as LSq, and this limitation must be considered with respect to the study objectives when choosing an experimental design. Cows that were continuously fed ADMP in RCBD were observed to have the lowest DMI throughout most of the study (Figure 1), although this observation was not statistically significant ($P = 0.122$; Table 4). Many studies (Lee et al., 2012a; Giallongo et al., 2016; Barros et al., 2017) and meta-analyses (Huhtanen and Hetta, 2012; Zanton, 2016) have shown that reducing dietary CP concentration leads to reduced DMI, although exceptions to this observation are also frequent (Wu and Satter, 2000; Colmenero and Broderick, 2006; Lee et al., 2012b). Responses in DMI that are due to hepatic oxidation of propionic acid are very rapid in the postpartum period (Maldini and Allen, 2018), whereas a prolonged timeframe for DMI response was apparently required when dietary protein level was changed in mid lactation (Law et al., 2009). The mechanism by which CP level affects DMI is not well understood. Frequently, a decrease in DMI due to decreasing CP level is attributed to lower ruminal N status resulting in increased

filling effects due to reduced NDF digestibility (Allen, 2000). Lower NDF digestibility was observed for cows fed LOMP in LSq (Table 5), and lower potentially digestible NDF digestibility with LOMP was observed in both designs ($P < 0.054$), although these responses did not result in differences in DMI. Recently, MP status was found to interact with supply of additional MP provided by casein infusion such that increasing MP at higher MP level resulted in reduced DMI, whereas the DMI increased when MP was deficient (Martineau et al., 2016). Ultimately, main effects on DMI were not significant in this study, whether due to competing mechanisms of the action of CP or due to other factors controlling intake under these conditions. The remaining effects on production, digestibility, and metabolism are therefore interpreted independent of treatment effects on DMI, which confounds many studies in protein nutrition (Huhtanen and Hristov, 2009).

Production of milk and milk components is shown in Table 4, and the time course of production responses for the treatment sequences are shown in Supplemental Figures S2 and S3 (<https://doi.org/10.3168/jds.2018-15730>). Irrespective of design, feeding LOMP resulted in lower milk, protein, and lactose yields than feeding ADMP, as was expected. Responses to changing MP level were very rapid for milk yield and milk protein yield, with a large proportion of the ultimate response occurring within 1 wk. This is reflected in the close similarity in the least squares means and treatment differences observed for RCBD and LSq. Production and endocrine responses to total diet restriction can be observed in as little as 2 d (Ferraretto et al., 2014); however, a physiological adaptation to this new level of energy and protein availability may be more prolonged and depend on the stage of lactation (Gross et al., 2011). The time course of production responses to postprandial protein or AA infusion has been observed in several studies to be very rapid, often occurring on the same

Table 4. Effect of protein concentration¹ and experimental design on LSM² of DMI, milk yield and composition, and BW in dairy cows fed according to a Latin square design (LSq) or a randomized complete block design (RCBD)

Item	Design	Diet		SEM	Difference	SED ³	P-value	
		ADMP	LOMP				Diet	Diet × week ⁴
BW, kg	RCBD	738	731	4.9	−7	6.7	0.301	0.669
	LSq	733	732	5.9	−1	4.3	0.843	0.650
BW change, kg/28 d	RCBD	7.9	4.9	1.92	−3.0	2.63	0.268	0.975
	LSq	4.9	9.2	3.77	4.3	5.97	0.478	0.767
DMI, kg/d	RCBD	28.9	30.4	0.65	1.5	0.91	0.122	0.031
	LSq	30.2	30.8	0.58	0.5	0.48	0.267	0.838
Milk, kg/d	RCBD	55.5	53.0	0.72	−2.5	0.98	0.018	0.582
	LSq	55.9	53.0	1.03	−2.9	0.91	0.004	0.402
Protein, kg/d	RCBD	1.62	1.54	0.020	−0.08	0.027	0.011	0.527
	LSq	1.65	1.54	0.032	−0.11	0.025	<0.001	0.799
Fat, ⁵ kg/d	RCBD	1.81	1.83	0.079	0.02	0.109	0.840	0.110
	LSq	1.88	1.81	0.056	−0.07	0.045	0.125	0.475
Lactose, kg/d	RCBD	2.72	2.59	0.038	−0.13	0.052	0.017	0.559
	LSq	2.76	2.61	0.052	−0.14	0.046	0.006	0.447
MUN, g/d	RCBD	4.74	3.17	0.181	−1.57	0.247	<0.001	0.007
	LSq	4.73	3.28	0.138	−1.45	0.119	<0.001	0.208
Protein, %	RCBD	2.92	2.92	0.027	0.00	0.036	0.999	0.112
	LSq	3.00	2.96	0.026	−0.05	0.021	0.032	0.078
Fat, ⁵ %	RCBD	3.18	3.57	0.117	0.39	0.165	0.026	0.223
	LSq	3.39	3.43	0.090	0.03	0.063	0.602	0.018
Lactose, %	RCBD	4.93	4.88	0.022	−0.04	0.030	0.167	0.630
	LSq	4.94	4.95	0.016	0.01	0.012	0.381	0.729
MUN, mg/dL	RCBD	8.61	6.06	0.299	−2.56	0.407	<0.001	0.002
	LSq	8.56	6.13	0.243	−2.43	0.194	<0.001	0.071
NE _L , Mcal/d	RCBD	36.70	35.88	0.844	−0.82	1.147	0.483	0.189
	LSq	37.63	35.80	0.722	−1.82	0.653	0.011	0.846
NE _L /DMI, Mcal/kg	RCBD	1.28	1.19	0.029	−0.10	0.040	0.025	0.008
	LSq	1.23	1.16	0.020	−0.07	0.020	0.002	0.757
Milk protein N/N intake, g/100 g	RCBD	33.56	34.63	0.631	1.07	0.868	0.230	0.053
	LSq	32.37	33.97	0.523	1.60	0.566	0.010	0.811

¹Diets contained either 16.5% CP for adequate MP (ADMP) or 14.6% CP for low MP (LOMP).

²Least squares means were covariately adjusted for initial production values measured during the 2 wk before feeding the treatment diets.

³Standard error of the difference.

⁴Interaction between diet and week, where week = 4, 8, 12, and 16 and is equivalent to periods 1 to 4 in LSq.

⁵Carryover effect of previous treatment was significant ($P < 0.05$) when design was LSq.

day or the days immediately following a change in the infusion rate (Broderick et al., 1970; Whitelaw et al., 1986; Metcalf et al., 1996). In contrast, several studies where cows were fed protected AA have shown more prolonged responses to AA supplementation, such as 2 wk (Benefield et al., 2009) or up to 5 wk (St-Pierre and Sylvester, 2005). Longitudinal studies that include a dietary change have also demonstrated that the duration of time on the new diet for the full response to take effect is around 2 to 5 wk (Wu and Satter, 2000; Law et al., 2009; Gross et al., 2011), although these studies were not designed to evaluate the time course of this response. However, if the treatment is severe enough over a long enough duration, the full response to a new diet may not be observed (Law et al., 2009).

As expected, MUN concentration and output were lower for LOMP than for ADMP, and this response was not affected by design. Lewis (1957) observed that BUN concentration in sheep adapted to changes in

dietary carbohydrate degradability or CP level within approximately 3 d, which was a similar time course that was observed when sheep were turned out on pasture (Annison et al., 1959). Gustafsson and Palmquist (1993) observed rapid diurnal equilibration between serum urea and milk urea in dairy cows. Taken together, these results correspond very closely to the time course response observed after diets were changed between periods in this study (Supplemental Figure S4, <https://doi.org/10.3168/jds.2018-15730>) and others (Barros et al., 2017).

After changing diets between periods 1 and 2, cows changing to ADMP had higher milk and milk protein production than cows that were maintained on that diet for periods 1 and 2; the opposite response was observed for cows changing to LOMP. This type of response could be reflective of a compensatory type of productivity resulting from a carryover in productive efficiency (Ferrell et al., 1986). However, these respons-

Figure 1. Temporal response to adequate MP (ADMP) and low MP (LOMP) when fed according to randomized complete block design (RCBD) or Latin square design (LSq). When fed in RCBD, there was a tendency for a linear $\times$ treatment interaction ($P < 0.09$) and a significant ($P < 0.05$) quadratic $\times$ treatment interaction for DMI. Treatment $\times$ time interactions were not significant ($P > 0.20$) for milk yield. Error bars are the SEM.

es were not observed with the dietary changes between periods 2 and 3, and neither the carryover effect of previous treatment nor the treatment $\times$ period interaction were significant for these production responses. These observations are consistent with the results of the 2 recent meta-analyses on the effects of experimental design on milk production when changing CP within the range of CP used in this study (Huhtanen and Hetta, 2012; Zanton, 2016).

Yield of milk fat was not affected by dietary treatment (Table 4) in either design ($P > 0.125$). Interestingly, carryover effects of previous treatment were observed due to the higher levels of milk fat production observed when LOMP was the previous treatment than when ADMP was the previous treatment. Rico and Harvatin (2013) evaluated the time course of diet-induced milk fat depression and recovery, and from these results it could be expected that milk fat depression could be

induced from altered ruminal biohydrogenation and can recover after 14 to 21 d, where samples in this study were analyzed at d 24 to 25. The previous treatment effects could alternatively reflect an altered partitioning of fat energy toward milk fat secretion when cows were previously nutritionally restricted due to having consumed LOMP compared with ADMP. Milk fat concentration was likewise affected by previous treatment in LSq, where cows previously fed LOMP had higher milk fat concentration than when previously fed ADMP, although the combination of a previous treatment effect for milk fat yield and no previous treatment effect for milk yield likely created a dilution effect for milk fat concentration. Inferences for milk fat concentration were quite different between designs, with cows fed ADMP in RCBD having reduced milk fat, whereas there was no effect in LSq. A tendency for milk fat concentration to decrease more with increasing CP in

Table 5. Effect of protein concentration¹ and experimental design on LSM² of intake and digestibility in dairy cows fed according to a Latin square design (LSq) or a randomized complete block design (RCBD)

Item	Design	Diet		SEM	Difference	SED ³	P-value	
		ADMP	LOMP				Diet	Diet × week ⁴
Intake, kg/d								
OM	RCBD	27.19	28.48	0.570	1.28	0.790	0.118	0.003
	LSq	28.29	28.89	0.462	0.60	0.494	0.239	0.943
CP	RCBD	4.87	4.50	0.101	−0.37	0.140	0.016	0.007
	LSq	5.08	4.57	0.078	−0.50	0.083	<0.001	0.971
NDF _{OM} ⁵	RCBD	8.36	9.58	0.182	1.21	0.252	<0.001	0.002
	LSq	8.68	9.72	0.147	1.04	0.160	<0.001	0.896
pdNDF _{OM} ⁵	RCBD	6.17	7.25	0.137	1.08	0.190	<0.001	0.004
	LSq	6.40	7.36	0.110	0.97	0.121	<0.001	0.890
Digestibility, %								
OM	RCBD	70.26	67.11	0.554	−3.15	0.796	<0.001	0.927
	LSq	70.14	67.28	0.668	−2.86	0.604	<0.001	0.200
Apparent CP	RCBD	64.38	57.57	0.809	−6.82	1.156	<0.001	0.264
	LSq	63.81	57.59	1.096	−6.23	1.033	<0.001	0.593
NDF _{OM}	RCBD	46.44	45.04	0.929	−1.40	1.323	0.301	0.945
	LSq	46.25	44.13	0.774	−2.12	0.963	0.038	0.357
pdNDF _{OM}	RCBD	63.25	59.66	1.230	−3.59	1.766	0.054	0.958
	LSq	63.04	58.52	1.028	−4.52	1.294	0.002	0.298

¹Diets contained either 16.5% CP for adequate MP (ADMP) or 14.6% CP for low MP (LOMP).

²Least squares means were covariately adjusted for initial production values measured during the 2 wk before feeding the treatment diets.

³Standard error of the difference.

⁴Interaction between diet and week, where week = 4, 8, 12, and 16 and is equivalent to periods 1 to 4 in LSq.

⁵aNDF_{OM} = neutral detergent fiber determined with α -amylase, Na₂SO₃, exclusive of ash. pdNDF_{OM} = potentially digestible aNDF_{OM} (difference between aNDF_{OM} and iNDF_{OM}). iNDF_{OM} = indigestible aNDF_{OM} based on 12 d in the rumen.

continuous trials was also observed in the meta-analysis of Huhtanen and Hetta (2012), although the magnitude of the difference between designs was greater in this study than in that analysis. However, in the analysis of Huhtanen and Hetta (2012) there were no differential effects in milk fat yield between designs.

The magnitude of production responses in milk protein and especially fat yield and the lower variability in LSq resulted in lower NE_L output for LOMP in LSq ($P = 0.008$) where differences were not observed in RCBD ($P > 0.40$). Despite these differences between designs in milk NE_L output and no differences between diets or designs in DMI, feed efficiency (NE_L/DMI, Mcal/kg) was significantly lower in LOMP than in ADMP in both designs (Supplemental Figure S5; <https://doi.org/10.3168/jds.2018-15730>). In contrast, N use efficiency (NUE; milk protein N/N intake, g/g) was higher in LOMP than ADMP, but this difference reached statistical significance only in LSq. Reducing dietary N is one of the most recognized and repeatable means by which to increase NUE (Huhtanen and Hristov, 2009; Powell et al., 2010; Lee et al., 2012b). However, from a practical standpoint, the improvement in NUE must be balanced against reduced milk protein production and potentially reduced feed efficiency (Zanton, 2016), although the joint effects of MP intake on feed and N efficiency are not very consistent (Phuong et al., 2013) and are likely dependent on changes in DMI and milk

fat yield, which are more variable than the responses in N intake and milk protein yield.

Digestibility and Metabolic Responses

Intake of OM was not affected by diet irrespective of design; however, intake of CP and NDF was different between diets irrespective of design as intended (Table 5). Diet × week interactions for intake of chemical components were observed, which was consistent with that observed for DMI where intake for LOMP was lower than ADMP for periods 1 to 3, with differences disappearing during period 4 (Figure 1). Organic matter and CP apparent digestibility were reduced for LOMP in both designs, whereas NDF and potentially digestible NDF digestibility was reduced for LOMP in LSq only. Many studies have shown that decreasing CP concentration in the diet decreases CP apparent digestibility (Broderick, 2003; Weiss et al., 2009; Lee et al., 2012b), and this effect is likely due to the dilution of endogenous fecal protein (Moran and Vercoe, 1972). Lower NDF digestibility with lower CP is also often observed (Broderick, 2003; Colmenero and Broderick, 2006; Lee et al., 2012b), although not always (Weiss et al., 2009). A typical recommendation for the time required for dietary adaptation for representative changes in digestibility is 7 to 14 d (Schneider and Flatt, 1975). There was no diet × time interaction for digestibility

measurements (Table 5); however, different inferences arose for NDF when LSq was the experimental design compared with RCBD, with the former resulting in a larger response than the latter with a smaller standard error. When potentially digestible NDF digestibility was evaluated, LOMP tended to have reduced digestibility compared with ADMP in RCBD ($P = 0.054$). Whether changes in the fibrolytic microbial population occur over a more prolonged timeframe than has typically been assumed is not known. Weimer et al. (2010) showed that cows require 63 d to revert to pre-exchange microbial communities upon near-total exchange of ruminal contents, although ruminal chemistry parameters reverted to pre-exchange values within a few days. More recent work by Weimer et al. (2017) showed that microbial communities resembled pre-exchange communities after only 10 d. When evaluating dietary changes, results from Machado et al. (2016) indicate that ruminal fermentation and microbial community composition stabilized after a maximum of 11 d. Likewise, Rico et al. (2015) observed that ruminal microbial populations responded rapidly to dietary changes between milk fat inducing and recovery diets. These results indicate that the time allowed during this study for ruminal microbial population changes should have been sufficient for a complete adaptation between diets and that the discrepancy between designs is more a reflection of higher between-animal variability for RCBD and higher statistical power for LSq.

Nitrogen responses to the different diets and designs are shown in Table 6. As intended, cows fed LOMP had lower N intake than cows fed ADMP, which resulted in lower urine N, milk N, urine urea N (UUN), and MUN excretion for LOMP, and these inferences were unaffected by design. Urea clearance, UUN:PUN ratio, or UUN:urine N ratio were lower for cows fed LOMP than for those fed ADMP, and these inferences were not affected by design. Fecal N was not different between diets. These combined measurements resulted in higher residual N in ADMP than in LOMP, although this was significant only in LSq ($P < 0.031$) and did not differ from zero for either design. The primary route of excess N excretion is through urinary urea excretion (Spek et al., 2013), and changes in N excretion through this pathway would be expected to be fairly rapid unless a change in N partitioning to or from body protein pools occurred (Paquay et al., 1972). Biddle et al. (1975a,b) fed growing steers diets with 6% CP to deplete the so-called labile N reserves for 5 wk and observed that urinary N excretion was minimized by 3 wk after the diet change. When the diet changed to a higher CP recovery diet, the growing steers reached the point of maximum urinary N after 2 wk on this new diet but ultimately continued on a pattern of decline after these

initial 2 wk. In growing ruminants postruminally infused with different amounts of protein, changes in N excretion and retention have been very rapid, typically stabilizing in less than 1 wk (Hovell et al., 1987; Liu et al., 1995; Schroeder et al., 2006). Based on these data and the results in the literature, N partitioning measured in ruminants responds rapidly to changes in MP level, and the inferences were generally unaffected by experimental design with lactating dairy cows in this study, although the residual N response differed in magnitude between designs.

Plasma metabolites and insulin are shown in Table 7. As expected, cows fed LOMP had lower PUN compared with ADMP, and these inferences were consistent between designs. As was previously indicated, BUN concentration responded rapidly to changing dietary conditions (Lewis, 1957; Annison et al., 1959). The concentrations of PUN observed in this study were higher than those observed at similar concentrations of CP in previous studies (Colmenero and Broderick, 2006; Lee et al., 2012b) and what would be predicted based on MUN concentration (Broderick and Clayton, 1997). The reason for this discrepancy is not apparent. Glucose and NEFA were not affected by treatment and had similar levels across designs. Insulin concentration was numerically lower for both designs when cows were fed LOMP; however, this response was significant only for LSq ($P < 0.025$). There was very weak evidence for a carryover effect of previous treatment for NEFA ($P < 0.09$) and insulin ($P < 0.17$). Plasma NEFA concentration was lower and insulin was higher when the previous treatment was LOMP than when the previous treatment was ADMP. This response would tend to indicate that cows previously fed LOMP were in an anabolic state and partitioning fat toward tissues that would be insulin responsive in the subsequent period compared with cows previously fed ADMP. However, this interpretation is not consistent with the greater milk fat concentration or yield for cows previously fed LOMP, which tends to be reduced in periods of higher insulin concentration (Grinari et al., 1997). Nevertheless, these plasma carryover responses are not significant but could be monitored or tested directly in future experiments related to MP nutritional history.

Overall Design Effects

Within the range of dietary protein changes made in this experiment, previous meta-analyses did not detect differences between experimental designs (Huhtanen and Hetta, 2012; Zanton, 2016). However, milk fat concentration tended to differ between designs in the former analysis, and different milk production and feed efficiency responses were observed at higher levels of

Table 6. Effect of protein concentration¹ and experimental design on least squares means² of N distribution in dairy cows fed according to a Latin square design (LSq) or a randomized complete block design (RCBD)

Item ³	Design	Diet		SEM	Difference	SED ⁴	P-value	
		ADMP	LOMP				Diet	Diet × week ⁵
Intake N, g/d	RCBD	779	720	16.2	−59	22.5	0.016	0.007
	LSq	812	731	12.6	−80	13.3	<0.001	0.971
Fecal N, g/d	RCBD	282	306	9.9	25	14.6	0.105	0.733
	LSq	293	310	12.6	18	9.8	0.088	0.655
Urine N, g/d	RCBD	238	187	7.9	−51	10.7	<0.001	0.867
	LSq	230	182	10.1	−47	9.3	<0.001	0.928
Urinary urea N, g/d	RCBD	181	104	6.1	−77	8.4	<0.001	0.011
	LSq	176	100	6.8	−76	6.6	<0.001	0.288
Milk protein N, g/d	RCBD	254	242	3.2	−12	4.3	0.011	0.527
	LSq	257	240	5.0	−17	3.9	<0.001	0.799
MUN, g/d	RCBD	4.74	3.17	0.181	−1.57	0.247	<0.001	0.007
	LSq	4.76	3.31	0.138	−1.45	0.119	<0.001	0.208
Residual N, g/d	RCBD	0.2	−14.9	8.43	−15.0	11.42	0.201	0.181
	LSq	24.2	−8.1	14.98	−32.3	14.00	0.031	0.894
UUN/urine N, %	RCBD	76.17	56.82	1.522	−19.35	2.069	<0.001	0.352
	LSq	77.93	56.84	1.849	−21.09	2.258	<0.001	0.025
UUN/PUN	RCBD	24.4	19.3	1.04	−5.2	1.41	0.001	0.344
	LSq	25.1	17.3	0.83	−7.8	1.03	<0.001	0.766
Urea N clearance, L/h	RCBD	31.8	24.8	1.16	−7.1	1.57	<0.001	0.241
	LSq	32.6	23.8	1.19	−8.8	0.99	<0.001	0.284

¹Diets contained either 16.5% CP for adequate MP (ADMP) or 14.6% CP for low MP (LOMP).²Least squares means were covariately adjusted for initial production values measured during the 2 wk before feeding the treatment diets.³UUN = urine urea N; PUN = plasma urea N.⁴Standard error of the difference.⁵Interaction between diet and week, where week = 4, 8, 12, and 16 and is equivalent to period 1 to 4 in LSq.

CP in the latter analysis. A critical weakness of both of these analyses was that cows in continuous studies had lower DIM than cows in changeover trials. To address this weakness, this study was conducted using a common population of cows fed different levels of MP and studied under common management and experimental conditions using LSq and RCBD experimental designs.

A graphical depiction of the inferences relative to the null hypothesis of no treatment difference is shown in Figure 2A. Overall, there is close concordance (concordance correlation coefficient = 0.83) and a strong relationship ($R^2 = 0.84$) between *t*-scores for LSq and RCBD. As discussed previously, there are several instances where the inference from LSq would yield a

Table 7. Effect of protein concentration¹ and experimental design on least squares means² of plasma measurements in dairy cows fed according to a Latin square design (LSq) or a randomized complete block design (RCBD)

Item ³	Design	Diet		SEM	Difference	SED ⁴	P-value	
		ADMP	LOMP				Diet	Diet × week ⁵
PUN, mg/dL	RCBD	24.05	17.71	0.613	−6.34	0.839	<0.001	0.312
	LSq	23.06	17.92	0.631	−5.14	0.791	<0.001	0.316
Glucose, mM	RCBD	3.09	3.05	0.060	−0.04	0.081	0.637	0.080
	LSq	3.03	3.07	0.077	0.03	0.059	0.564	0.045
NEFA, ⁶ μM	RCBD	139.63	140.57	5.722	0.94	7.768	0.905	0.608
	LSq	145.68	145.99	3.345	0.31	5.913	0.959	0.013
Insulin, pM	RCBD	118.91	105.31	7.808	−13.60	10.996	0.229	0.203
	LSq	129.09	102.85	8.662	−26.25	10.916	0.025	0.278

¹Diets contained either 16.5% CP for adequate MP (ADMP) or 14.6% CP for low MP (LOMP).²Least squares means were covariately adjusted for initial production values measured during the 2 wk before feeding the treatment diets.³PUN = plasma urea N; NEFA = nonesterified fatty acids.⁴Standard error of the difference.⁵Interaction between diet and week, where week = 4, 8, 12, and 16 and is equivalent to periods 1 to 4 in LSq.⁶Carryover effect of previous treatment approached significant ($P < 0.09$) when design was LSq.

rejection of the null hypothesis of no effect on the basis of $P < 0.05$, whereas the test for RCBD was not rejected based on these data. There were no instances in which the inferences about treatment effects were reversed in LSq compared with RCBD. Therefore, LSq provided stronger evidence against the null hypothesis than RCBD when LOMP is estimated to be lower than ADMP; however, the direction and significance of the inference would not be affected. Only in the case of milk fat concentration was the inference for RCBD to reject the null hypothesis where the LSq null hypothesis could not be rejected on the basis of $P < 0.05$. Overall, these responses highlight the capability of LSq to detect smaller differences than RCBD due to making within-cow comparisons in LSq. This would be the expectation if responses in LSq replicated treatment responses observed when diets were fed for a longer duration as in RCBD.

Although the statistical tests of the data are necessary for understanding the strength of the evidence for the hypothesized model, they are not sufficient because the magnitude of the responses is required to make a nutritional or management decision. Figure 2B shows the relationship between LSq and RCBD for the relative responses between LOMP and ADMP least squares means: $(\text{LOMP} - \text{ADMP})/[0.5 \times (\text{ADMP} + \text{LOMP})]$. Body weight change and residual N resulted in a large and opposite response when estimated within the different designs; for clarity, these results are excluded from the following discussion but are addressed later. Overall, there is close concordance between the numerical results of LSq and RCBD relative differences (concordance correlation coefficient = 0.97). The results from Figure 2B are shown as a Bland-Altman plot (Bland and Altman, 1986) in Figure 2C along with the mean bias and the upper and lower limits of agreement between designs. There was no detectable slope bias between designs ($P > 0.50$); however, there was significant negative mean bias, indicating that over all of the responses measured in this study, the relative response observed in LSq was 1.84 percentage unit lower than the relative response observed in RCBD ($P < 0.026$). A few specific responses stand out with the relative response estimated to be lower in LSq than in RCBD: milk fat percentage, insulin concentration, and the UUN:PUN ratio. The nature of these individual responses between designs and the large opposite responses for BW change could be reflective of continued alteration in energy partitioning after a shorter time on treatment when changing between diets compared with when fed the same diets continuously, although most responses show reasonably good agreement between designs.

This experiment was designed to have treatment responses as extreme as practically relevant. Therefore,

the results of this study are limited to these extreme treatment differences, and an extrapolation to a scenario of lower treatment differences must be done with extreme caution. The reason for designing the diets to have extreme treatment responses was 2-fold: (1) there had to be treatment differences to be able to understand whether the inferences from this experiment are affected by design, and (2) this is where previous meta-analyses have shown these designs to yield different inferences (Huhtanen and Hetta, 2012; Zanton, 2016). The inferences of these meta-analyses were based on many experiments with different objectives, diets, sampling intensities, cow DIM, and so on. This study was conducted to directly test the effect of experimental design with animal characteristics, diets, and sampling that were the same between designs. The consistency of the conclusions from this study and those of the meta-analyses under hypothesized extreme treatment differences strengthens the inferences of the meta-analyses under situations of lower treatment differences that cover a greater inferential space.

CONCLUSIONS

In this experiment, where MP concentration was formulated to promote large treatment differences, diets resulted in different levels of production and N metabolism that were consistent with literature responses in both direction and magnitude. Under the conditions of this experiment with multiparous early-lactation cows fed diets with different levels of MP, the majority of response variables measured for N metabolism, digestibility, and production responses were not affected by experimental design. Treatment responses that may respond differently over time (e.g., DMI in this study) and that represent altered nutrient partitioning (e.g., BW change and residual N in this study) will likely not be reflected in short changeover designs. Milk fat yield and percentage responses were affected by previous treatment carryover in LSq. Collectively, it is concluded that most responses to differences in MP are consistent between LSq and RCBD; however, differing responses in energetic variables require further investigation.

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Figure 2. (A) Inference graph for differences between low MP (LOMP) and adequate MP (ADMP). Values for t -scores where $P < 0.05$ are shown as the vertical and horizontal lines; values more extreme than these cutoffs indicate statistical significance under the threshold defined in this experiment. Values lying within the shaded sections represent making the same inference about the direction of the treatment response across designs. Dashed line is the best fit linear response: randomized complete block design (RCBD) = 0.021 (0.274) + 0.65 (0.047) × Latin square design (LSq); $R^2 = 0.84$. Diagonal line is $y = x$; concordance correlation coefficient (95% CI in parentheses) = 0.83 (0.738, 0.897). (B) Relative response between LOMP and ADMP LSM. For clarity, the values for BW change and residual N are not shown, which had the coordinates (-46.87, 60.99) and (205.44, -401.24), respectively. Dashed line is the best fit linear response exclusive of BW change and residual N: $RCBD = 1.55 (0.814) + 1.00 (0.043) \times LSq$; $R^2 = 0.94$. Diagonal line is $y = x$; concordance correlation coefficient (95% CI in parentheses) = 0.97 (0.934, 0.982). (C) Difference plots for the relative response between LOMP and ADMP LSM without the response in BW change (108.87, 7.06) and residual N (-606.68, -97.90). Slope bias was not detected ($P > 0.50$); mean bias (95% CI in parentheses) = -1.84 (-0.24, -3.46) was significant (dashed line; $P < 0.026$). The limits of agreement (mean bias $\pm 2 \times SD$) ranged from -9.44 to 6.25. UUN = urine urea N; PUN = plasma urea N.

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