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Crude protein oscillation in diets adequate and deficient in metabolizable protein: effects on nutrient digestibility, nitrogen balance, plasma amino acids, and greenhouse gas emissions

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ABSTRACT

Reducing dietary crude protein (CP) is a well-established means to improve N use efficiency. Yet, few studies have considered if transient restrictions in dietary CP could reduce the environmental footprint of late lactation cows. We hypothesized that the effects of CP feeding pattern on digestibility and environmental outputs would be amplified at lower dietary CP. We tested CP levels below and near predicted requirements (LP, 13.8%; HP, 15.5%) offered in 2 feeding patterns: where diets alternated ± 1.8 percentage units CP every 2 d (oscillating; OF) or remained static (SF). Our study used a 2x2 factorial design with 16 mid- to late-lactation Holsteins (M = 128, SD = 12 DIM), divided into rumen-cannulated (n = 8) and non-cannulated subsets (n = 8). For each 28-d experimental period, we recorded feed intake and milk production and took samples of orts (1x/d) and milk (2x/d) for 4 d. For the cannulated subset, we measured and sampled from the total mass of feces and urine production and collected plasma 2x/d across 4 d. For the non-cannulated subset, we sampled carbon dioxide and methane emissions 3x/d for 4 d. For each subset, we fit linear mixed models with fixed effects for CP level, CP feeding pattern, the interaction of CP level and CP feeding pattern, period, and a random effect for cow. For plasma and urinary urea-N, we conducted time series analysis. Contrary to our hypothesis, we found no evidence that dietary CP level and CP feeding pattern interacted to influence N balance, nutrient digestibility, or gas emissions. Results showed HP resulted in similar milk N but increased manure N, reducing N use efficiency (milk true protein N/intake N) relative to LP. For OF, urea-N in urine and plasma peaked 46–52 h after the first higher-CP phase feeding. Nutrient digestibility and gas emissions were similar across treatments, except CO_2 production was greater for OF-HP. In summary, measured variables were minimally affected by dietary CP alternating ± 1.8 percentage units every 48 h, even when average dietary CP was fed below predicted requirements (LP). Although our findings suggest that mid- to late-lactation cows are resilient to oscillation in dietary CP, oscillating CP neither reduced the environmental footprint by improving nutrient use efficiencies nor reduced the potential for direct and indirect greenhouse gas emissions.
Keywords: dairy cow, methane, N balance, protein oscillation

INTRODUCTION

Efforts to optimize lactation performance while managing environmental impacts of N have centered on reducing dietary CP while supplying adequate AA, energy, and other nutrients to support milk protein synthesis. The most well-established method to improve N use efficiency (NUE; milk true protein N / Intake N) in lactating cattle is the reduction of dietary CP (Dijkstra et al., 2013). Reducing dietary CP has been shown to enhance urea-N recycling to the gastrointestinal tract (GIT), reduce renal urea-N clearance, and improve postabsorptive N efficiencies by altering the AA affinities of various tissues including those of the mammary gland (Lapierre and Lobley, 2001; Rius et al., 2010; Sinclair et al., 2014). Additionally, research showed a close linear relationship between dietary CP intake and urinary urea-N concentration (UUN) and rate of excretion (UUNY), which indicates that dietary CP is an important contributor to the amount of reactive N in manure (Powell et al., 2011). As lactation advances, changes in DMI, milk and component production, and metabolic state may affect N partitioning differently depending on the dietary CP level (Letelier et al., 2022). Although extensive research has evaluated N balance associated with different dietary CP levels given certain cow characteristics, most studies assessed responses after adaptation to diets formulated

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for constant composition over time. It remains unclear if the N-sparing effects observed with long-term dietary CP reduction could be achieved with transient restrictions in dietary CP intake, for example, by alternating dietary CP over time in an oscillating pattern.

Sheep and beef cattle fed oscillating CP levels maintained performance and sometimes retained a greater proportion of dietary N relative to control animals fed CP with a static pattern (Ludden et al., 2003; Schauer et al., 2010). Limited research on mid- to late-lactation dairy cattle showed that feeding oscillating CP ± 1.5 to 3.0 percentage units of DM at 24- to 48-h intervals had minimal effects on productive performance, inconsistent effects on NUE, and minimal or positive effects on digestibility of CP and other nutrients (Brown, 2014; Kohler, 2016; Tebbe and Weiss, 2020; Rauch et al., 2021). These studies used a variety of different diets, ranges of CP oscillation, nutrients substituted for CP, and time intervals for oscillation. Several notable findings have contributed to mechanistic understanding of oscillating diets. Kohler (2016) found greater apparent ruminal DM and OM digestion and a lesser amount of total N passage to the omasal canal for cows fed oscillating-CP patterns. Tebbe and Weiss (2020) showed no differences between oscillating and static-fed cows in BW or composition, and in plasma concentrations of glucose, insulin, and most AA. However, it remains unclear if the effects of oscillating CP depend on the average level of dietary CP, i.e., if oscillating CP is more effective at lower dietary CP. Additionally, limited research has described the effects of CP level and CP feeding pattern on greenhouse gas (GHG) production, for example, through digestibility-mediated effects (Benchaar et al., 2023; Hynes et al., 2016). Therefore, our objective was to evaluate the effects of dietary CP oscillation on nutrient digestibility, N balance, plasma AA, and GHG emissions. We hypothesized that oscillating dietary CP would enhance digestibility, reduce N excretion in manure, and increase methane (CH_4) production of mid- to late-lactation cows when the average dietary CP was below predicted requirements, but not when CP was fed near predicted requirements.

MATERIALS AND METHODS

This study was conducted from April to August 2021 at the University of Wisconsin—Madison Dairy Cattle Center. All procedures involving animals were approved by the University of Wisconsin–Madison Institutional Animal Care and Use Committee (protocol #A006439).

Animals and Experimental Design

We used 16 multiparous, mid- to late-lactation Holstein cows (mean = 128, SD = 12 DIM when the experiment began). Cows were divided into 2 subsets: non-cannulated (n = 8; mean = 122, SD = 11 DIM) and cannulated (n = 8; 10 cm ruminal cannula, Bar Diamond Inc., Parma, ID; mean = 135, SD = 9 DIM). Milk production, BW, BCS, and DMI were recorded for all cows and used to calculate N and feed efficiency metrics. The cannulated subset of cows was used for total urine and feces collection and plasma sampling. Due to GHG emission headbox procedures (described below) and possible gas escape through the ruminal cannula, only the non-cannulated subset was used for GHG emission measurements. Within each subset, we assigned cows to 4 treatment sequences in a Latin Rectangle arrangement. Treatments constituted a 2x2 factorial arrangement with 2 levels of CP (LP = 13.8, HP = 15.5% CP of DM) and 2 CP feeding patterns (OF = oscillating dietary CP ± 1.8 percentage units with diet changes at 48 h intervals, SF = static dietary CP). Each 28-d experimental period had an adaptation and GHG equipment re-training period (d 1–14), a GHG measurement period (d 14–21), and a 4-d intensive sampling period (d 25–28). Throughout the experiment, cows were housed in individual tie stalls with rubber mats. Stalls were bedded with wood shavings except during total fecal and urine collection. Cows were milked twice daily (0400 and 1600 h) and fed a TMR once daily (0800 h) aiming for a 5% refusal rate. Feed was pushed toward cows in the bunk once daily (~1800 h). Cows had *ad libitum* access to automatic waterers. The ambient temperature was controlled with an evaporative tunnel ventilation system. Milk and production performance was described in a separate manuscript (Erickson et al., 2023).

Dietary Treatments

Full ingredient and nutrient composition of diets is available in a separate manuscript (Erickson et al., 2023). We formulated LP to supply less than predicted requirements for RDP and MP and HP to supply adequate RDP and MP (NRC, 2001). Each OF CP feeding pattern alternated between 2 diets (OF-LP 12.2–15.5%, OF-HP 13.8–17.3% CP) every 48 h throughout the experimental period such that mean diet composition equaled that of corresponding SF treatments. Each SF treatment consisted of a single diet fed throughout the experimental period (SF-LP, 13.8%; SF-HP, 15.5% CP). All diets were delivered as a TMR and had a constant 60:40 forage-to-concentrate ratio (DM basis) with dietary differences implemented using 4 different con-

centrate formulations. In the concentrate formulations, soybean hulls, ground corn, and expeller soybean meal were exchanged with solvent soybean meal to target constant dietary NDF to starch and rumen-degradable protein to CP ratios.

Measurements, Sampling, Laboratory Analysis, and Calculations

Unless otherwise stated, laboratory analysis occurred at the USDA Dairy Forage Research Center in Madison, WI.

Milk, Feed, Orts, BW, and BCS. Procedures for sampling, laboratory analysis, and aggregation methods for milk, TMR, forages, and orts are detailed in a separate manuscript (Erickson et al., 2023). In brief, milk weights were measured using the parlor system and recorded on paper by farm staff. Milk samples were taken via automatic samplers, preserved with bronopol tablets, and refrigerated until shipment for analysis. Milk samples were transported to a commercial laboratory for spectrometric analysis (Foss FT6000; Foss Electric, Hillerød, Denmark; AgSource Laboratories, Verona, WI). Milk N was calculated by the amounts (g) of milk true protein N and milk urea N, using a true protein to N conversion factor of 6.38. We took daily samples of TMR (n = 4), forages (n = 2), and orts (n = 8) from d-25 to d-28 of each experimental period. Compositing procedures for feeds and orts are detailed in a separate manuscript (Erickson et al., 2023). Feed and orts samples were dried at 105°C for 24 h to determine DM. The NDF procedure used a neutral detergent solution with amylase and sodium sulfite (method 2002.04.2005; Mertens, 2002). Both NDF and ADF residues were ashed at 600°C for 2 h to determine NDFom and ADFom (method 973.18, AOAC International, 1996). Indigestible NDF (iNDFom) was determined after incubating duplicate 500 mg samples in F57 polyester filter bags (25 micron porosity, 5x5 cm) for 240 h in the rumen of 2 cows fed a diet similar to experimental diets (major ingredients: alfalfa haylage, corn silage, corn grain). Procedures for measuring, validating, and aggregating BW and BCS were detailed in a prior publication (Erickson et al., 2023). In brief, BW was recorded before feeding and immediately after the 0400-h milking for 4 d per experimental period. Three individuals scored body condition in 0.25 increments on a 1 to 5 scale on d 23 to d 28 of each experimental period.

Total collection of feces and urine. The total output of feces and urine were collected for the subset of cannulated cows (n = 8) during the intensive sampling period (d 25–28). Feces and urine were weighed and sampled 3 times daily at 0400, 1200, and 2000 h. Each cow's feces was collected into a custom-made galvanized steel pan set beneath a grate in a gutter behind her stall. During total manure collections, wooden dividers (1.2 × 2.4 m) were bolted to partitions to span the full length of the stall to separate each cow's fecal material. However, windows at the front of the divider allowed regular social behavior. Feces were scraped into the gutter pan regularly throughout the day. At each sampling time point, a cow's feces was shoveled into a plastic bin and weighed on a floor scale (CPWPLUS 150M, Adam Equipment, Oxford, CT). After mixing with a shovel, a subsample (100–150 g) was collected into a specimen cup. Feces samples were weighed immediately and dried at 55°C for 96 h for sample preservation. Urine was collected by bladder catheterization from d 24–28 each experimental period and recorded from d 25–28 (26 French, 75 mL Foley balloon lubricious catheter, C.R. Bard Inc., Covington, GA). For each 8-h interval, we acidified polyethylene urine carboys with 300 mL sulfuric acid (Item #13891, United States Plastic Corp., Lima, OH). At each sampling time point, we poured urine into a bucket to weigh on the same floor scale as feces, stirred it thoroughly, and sampled 100–150 mL into a specimen cup. Each urine sample was pH tested using a portable pH meter (WTW 3110 m; WTW, Xylem, Rye Brook, NY; M = 2.8, SD = 1.3). Urine subsamples (2.0 mL) were diluted with deionized water (8.0 mL), mixed, and stored in conical tubes at −20°C. In the event of catheter expulsion, all feces and urine measurements for that cow and time point were discarded.

Before analysis, dried feces samples were composited by cow and period with fecal sample DM weighted by fecal DM output per time point (n = 30 due to 2 missing cow-periods). To minimize analytical variation in components of N balance, total N in feeds, feces, and urine was determined with the same Dumas combustion method and equipment (Leco FP-2000 N Analyzer; Leco Corp., St. Joseph, MO; AOAC method 990.03; AOAC International, 2006). Milk N was analyzed separately, as described above. Urine samples were thawed for 24 h at 4°C and analyzed for urea and creatinine with a flow-injection analyzer (Lachat Quik-Chem 8000 FIA; Lachat Instruments, Milwaukee, WI) using colorimetric and picric acid methods, respectively (Zanton and Hall, 2022). To determine apparent digestibility, feces samples were analyzed using the same methods as feeds described above.

Urine output per time point was calculated by correcting recorded urine weights for the weight of sulfuric acid. The yields of urinary N, UUN, and creatinine were first determined per time point by multiplying the sample concentration by the urine volume, then these values were aggregated to the day- and period-

level means. Similarly, feces output per time point was converted to a DM basis by multiplying the recorded feces output (kg as-is) by the proportion of DM in its respective sample. Manure output was calculated by summing urine and feces output (kg as-is). The urea clearance rate (UCR; L/min.) was calculated as UUNY (mg/d) divided by the mean plasma urea N (PUN; mg/dL) and converted to L/min. Apparent digestibility was calculated by the difference between nutrient ingestion and excretion. Potentially-digestible aNDFom (pdNDFom) was calculated by the difference between aNDFom and iNDFom.

Plasma. For the cannulated subset, we collected blood from the coccygeal vessels twice daily at 0700 and 1900 h from d 25–28. Samples of approximately 8–10 mL were collected into evacuated glass tubes containing 12.15 mg of K_3 EDTA or 158 USP sodium heparin (BD Vacutainer; Franklin Lakes, NJ), inverted several times, and placed in ice. After centrifuging samples at 1200 x *g* for 10 min at 4°C, we pipetted the plasma supernatant into 3 aliquots in 2 mL polypropylene microcentrifuge tubes and stored at −20°C. Before analyzing PUN in sodium heparin preserved samples (n = 240), we diluted each sample 1:1 by volume with trichloracetic acid (5% wt/vol), vortexed, and centrifuged for 10 min at 12,100 x *g* (MiniSpin; Eppendorf, Hamburg, Germany) to precipitate protein. Plasma urea-N in the supernatant was assayed with the QuantiChrom Urea Assay Kit (BioAssay Systems, Hayward, CA) and quantitated on an Eon Microplate Spectrophotometer (BioTek, Winooski, VT).

EDTA-preserved plasma samples were composited by equal volumes within cow by period and by oscillation phase (n = 60) and prepared for AA analysis by thawing on ice, gravimetrically adding labeled (^{13}C, ^{15}N) canonical 20 internal standard AA mix (Cambridge Isotope, Tewksbury, Massachusetts), and precipitating protein with methanol. After centrifugation, each sample supernatant was diluted in sodium phosphate and applied to a Dowex^{TM} 50W cation exchange resin 1 mL plug (Bio-Rad, Hercules, CA) and eluted with 3N ammonium hydroxide into glass tubes. The eluent was lyophilized overnight (Sentry 2.0 VirTis, SP Scientific) and the dried samples were dissolved in ethanol then transferred to microcentrifuge tubes. The solvent was removed under vacuum at 30°C (VacufugePlus, Eppendorf, Hamburg, Germany). AA analysis followed a procedure modified from Zheng (2015). Samples were derivatized using phenyl isothiocyanate, ethanol, and triethylamine, and after 20 min excess reagents were removed under vacuum at 45°C. Derivatized AA concentrations were measured on a Shimadzu Nexus LC-MS chromatography system equipped with a Phenomenex Kinetex C18 column (4.6x150 mm, 1.6 μm, Torrance, CA). The mobile phases were (A) 12.5 mM ammonium acetate, pH 6.5 and (B) Acetonitrile (70%):A (30%) and were used with the following gradient: 0–1.5 min, 5–10% B;1.5–9 min, 10–40% B ; 9–10.5 min, 40–100% B; 10.5–18 min, 100% B; 18–19 min, 100–5% B; and equilibrated at 19–23 min, 5% B. Flow rate was 0.8 mL/min and the column temperature was 40 C. Samples were dissolved in mobile phase A:B (90:10). Injection volume was 10 uL. Mass detection (Shimadzu MS 2020) of the derivatized AA was performed using ESI(+). Standard curves with AA concentrations of 10 – 675 uM were determined using canonical 20 AA (Cambridge Isotope, Tewksbury, Massachusetts) spiked with the same internal standards as those used for the samples.

Gas emissions. We sampled O_2, CO_2 and enteric CH_4 from the subset of non-cannulated cows using a GreenFeed (C-Lock, Rapid City, SD) headbox. The bait feed pellets included corn (90% of DM) and molasses (10% of DM). Through most of the experiment except during gas emission measurement, the bait feed was included in the TMR at 2% of dry matter. Cows were trained before starting the experiment and re-trained during d 7–14 of each experimental period. During training periods, the GreenFeed headbox was span-calibrated with pure gases. During d 14–21 of each period, we selected 4 d to sample gas production 3x/day to cover the intervals (−2.5)-(−0.5), 1–3, 4–6, 6.5–8.5, and 11–13 h relative to the 1x daily morning feeding with 12 samplings per cow per period. These time points were selected to over-sample (2x) the interval immediately before feeding so aggregate results would approximate daily CH_4 production across expected diurnal and feeding-related variation (Sun et al., 2019). At each sampling time point, we removed TMR in front of a cow's stall, dispensed approximately 300 g bait feed in 3 portions with the GreenFeed, and measured gas emissions for 5–8 min. Most (97.4%) of cow-time points resulted in successful measurements. For training and sampling days, the bait feed was withheld from the TMR, split into 3 feedings of 300 g, and fed in the GreenFeed unit (non-cannulated subset) or as a top-dress (cannulated subset). Due to broken equipment, we were unable to sample gas emissions in the second experimental period. We extended the experiment 28 d to collect missing observations and changed cows back to diets used for the second experimental period. The ratio of CH_4 to CO_2 was calculated on a liter per liter basis as in Madsen et al. (2010). The respiratory quotient was calculated as the quotient of the volumes (L) of CO_2 emitted and O_2 consumed, assuming densities of each gas at standard temperature and pressure.

Statistical Analysis

Missing data imputation. Two cows were removed from the study after contracting toxic mastitis, resulting in missing data for 2 cells (cow-periods) in the Latin Rectangle design for the cannulated subset. An additional cannulated cow was substituted into the design for period 3–4 after a toxic mastitis case in period 2. We considered these 2 cells missing completely at random and modeled only the cells with available data. In addition to major missing data (2 cow-periods with no available data), technical issues such as catheter expulsion resulted in missing data at the cow-period-time point level. We documented a small percentage of missing observations for milk weights and milk samples (0–2%), rumen fluid (2.6%), urine masses (5.8%), fecal masses (5.3%), urine samples (7.5%), and fecal samples (1.1%). To prevent biasing due to imbalance across time, for minor missing data we imputed missing values using stochastic regression. The imputation model contained fixed effects and interactions for known experimental design factors including period (1, 2, 3, 4), sampling time point (0400, 1200, 2000), and cow (i = 1 to 8). To counteract variance attenuation, each predicted value was augmented with a random draw from the observed residual distribution (Little and Rubin, 2002).

Modeling approach. To analyze overall differences due to treatment, we modeled the mean of observed values for a given cow and period using a linear mixed model with fixed effects for experimental period (E_j, where j = 1, 2, 3, 4), dietary CP level (P_k, where k = LP, HP), CP feeding pattern (F_l, where l = OF, SF), and the interaction term between CP level and CP feeding pattern (PF_{kl}). We included a random effect of cow (C_i, where i = 1 to 8) and a residual error term representing the n = 30 observations A$\left(\epsilon_{ijkl}\right)$.

$$\left(\epsilon_{ijkl}\right).$$

For the cannulated subset, we modeled faster-responding variables over time (urine analytes, PUN) to test for differential CP feeding pattern effects across sampling day. In these models, we added fixed effects and all possible interactions for day (m = 25, 26, 27, 28) and hour of sampling (n = 0400, 1200, or 2000 h for urine; n = 0700 or 1900 h for plasma) with treatments, and allowed the intercept to vary based on cow, period within cow, and day within period within cow, creating a block diagonal variance-covariance matrix. Given the 2 missing cells, the error term $y_{ijkl} = \mu + E_j + P_k + F_l + PF_{kl} + C_i + \epsilon_{ijkl}$ describes n = 360 urine or n = 240 PUN observations. The model for plasma AA was similar, except that the only time variable was oscillation phase (categorical, the 48-h interval for higher or lower CP). Additionally, the plasma AA model included only a random effect for cow, because a more complex random effect structure produced singular fits.

$$\left(\epsilon_{ijklmn}\right)$$

We conducted all data analysis using R version 4.1.2 (R Core Team, 2021). We considered $P < 0.05$ significant and $0.05 \leq P \leq 0.10$ tendencies. When standard errors differed due to imbalance between cells, we reported the greatest standard error. We fit models using the lme4 and lmerTest packages (Bates et al., 2015; Kuznetsova et al., 2017) using restricted maximum likelihood. We computed Type III sums of squares using afex (Singmann et al., 2022) and least squares means using the emmeans package (Lenth, 2016). To test for temporal patterns in dynamic N variables, we examined the interactions of CP level and CP feeding pattern with sampling day.

RESULTS AND DISCUSSION

Our study examined nutrient digestibility, N balance, NUE, N-containing plasma metabolites, and gas fluxes associated with 2 levels of dietary CP (LP, HP) and 2 CP feeding patterns (OF, SF). Predictions from the NASEM (2021) model presented in an earlier paper (Erickson et al., 2023) indicated that energy was oversupplied in all experimental diets, and MP supply was centered at 94% (LP) and 104% (HP) of requirements. In the oscillating feeding pattern, varying dietary CP concentration ± 1.8% of DM resulted in diets where MP supply spanned 83 to 104% (OF-LP) and 94 to 114% (OF-HP) of predicted requirements. Retrospective evaluation of dietary treatments with an updated nutritional model showed that efficiencies of Met and His exceeded targets for both LP and HP, suggesting that these AA were most limiting (Appendix Table A1; NASEM, 2021). The patterned variation in dietary CP imposed experimentally by oscillating feeding may reveal insights above the effects of variation in diet composition, informing precision nutritional management. Because we tested the effect of CP feeding pattern at multiple levels of CP, our research contributed to understanding potential interactions between CP level and CP feeding pattern. We hypothesized that CP feeding pattern would maintain production, enhance digestibility, and reduce environmental outputs of mid- to late-lactation cows for LP but not HP. For most variables, we found no evidence for an interaction between CP level and CP feeding pattern.

Nutrient Digestibility and Manure Output

Apparent total-tract nutrient digestibility and manure output results are shown in Table 1. Except for CP intake, we found no effects of CP level on nutrient intake and digestibility. Similar intake of DM, OM, aNDFom, and pdNDFom across LP and HP indicated that our diets successfully increased N intake (Table 2) with minimal alterations in DMI and the dietary carbohydrate fraction. Considering that dietary changes in NDF, starch, and CP in our trial were moderate, we did not expect differences in digestibility due to CP level. A meta-analysis by de Souza et al. (2018) found only a slight (0.59 percentage units) depression in NDF digestibility associated with 1 percentage unit greater starch content holding other factors constant. Previously, Aguerre et al. (2016) reported that increasing CP from 15.3 to 16.6% of diet DM increased DM, OM, and CP digestibility and had no effect on NDF digestibility. Using more extreme N-deficient diets than our trial, Belanche et al. (2012) reported that increasing dietary CP from 11 to 14% (80 vs. 110% of digestible N requirement) increased OM digestibility with concurrent shifts in the rumen microbial composition. Importantly, greater dietary CP generally increases apparent CP digestibility due to the dilution of metabolic fecal protein (NRC, 2001). In our trial, the modest predicted deficiency of MP had few effects on nutrient digestibility overall.

Neither nutrient intake nor digestibility differed due to CP feeding pattern, which was contrary to prior research. Several recent authors observed that oscillating CP feeding patterns increased digestibility of CP (Tebbe and Weiss, 2020; tendency) and DM, OM, CP, NDF, and starch (Rauch et al., 2021), yet failed to improve milk and component production. In these studies, dietary CP was primarily altered by replacing soybean meal with soy hulls and corn grain (Tebbe & Weiss, 2020) or with wheat and ground corn (Rauch et al., 2021). These authors suggested that oscillating CP feeding pattern may have decreased post-absorptive nutrient efficiency through unclear mechanisms. Importantly, DMI and N intake were either reduced (Tebbe and Weiss, 2020) or similar (Rauch et al., 2021) for oscillating vs. static CP feeding pattern. When replacing soybean, canola, and corn gluten meal with rolled barley and ground corn grain, Kohler (2016) found that oscillating CP feeding pattern increased DM ruminal digestibility and tended to increase OM ruminal digestibility relative to static. However, oscillating CP had no effects on total-tract apparent digestibility of DM, OM, CP, NDF, or ether extract (Kohler, 2016). In our trial, the lack of CP feeding pattern or CP-level by feeding pattern interaction on nutrient intake and digestibility does not rule out mechanistic differences in digestion. Still, our results indicated no compensatory gains in CP or OM digestibility resulting from the 48-h oscillating diets.

The range of manure output in our trial was similar to that observed on similar diets in past research (Wattiaux and Karg, 2004; Nennich et al., 2006) and predicted output based on DMI (NASEM, 2021). Output of manure tended to increase with HP, driven by an increase in urine output with HP. Conversely, CP level had no effect on output of feces or fecal DM. Previous work attributed greater urine volume to increased voluntary water consumption at greater CP intake (Van Vuuren and Smits, 1997; Sannes et al., 2002; Broderick, 2003). In our trial, CP feeding pattern had no effect on outputs of manure, urine, feces, or fecal DM, regardless of dietary CP level. In agreement, Tebbe and Weiss (2020) showed that free and feed water intake did not differ due to CP feeding pattern. Likewise, Kohler (2016) reported no differences in fecal DM or urine due to CP feeding pattern. Collectively, these results support the contention that oscillating CP feeding patterns do not alter urine or manure output on average.

N Balance

Results for N balance for the subset of cannulated cows are shown in Table 2. In general, the amounts and percentages of N in excreta were consistent with prior research (Olmos Colmenero and Broderick, 2006; Lee et al., 2019). Crude protein level influenced N partitioning in several ways. As designed, N intake was greater with HP and unaffected by CP feeding pattern. Milk N output was unaffected by CP level, but feeding HP diets increased urine N output and tended to increase fecal N output. This suggests the additional CP consumed and digested in HP diets exceeded capacity for milk protein synthesis and instead was directed primarily to manure N excretion. This is consistent with recent research showing a lack of milk protein response to additional dietary CP in late lactation (Mutsvangwa et al., 2016; Barros et al., 2017; Letelier et al., 2022). In our trial, the difference in urinary N output for LP and HP diets was primarily accounted for by additional UUNY (93.5%), comparable to values of 96–100% reported in prior work which examined differences between CP levels greater than those in our trial (Wattiaux and Karg, 2004). It is well-established that decreasing dietary CP has the potential not only to improve the efficiencies of MP and AA use by tissues (Lapierre et al., 2007; NASEM, 2021), but also to conserve urea-N by lessening its glomerular filtration and enhancing its reabsorption in the kidney (Müller et al., 2021; Souza et al., 2021). Interestingly, we found UCR was lower with LP. This is

Table 1. Apparent nutrient digestibility and manure output for cows fed diets varying in crude protein (CP): low protein (LP = 13.8% CP) or high protein (HP = 15.5% CP), with oscillating (OF, ± 1.8% CP at 48-h intervals) or static (SF) feeding patterns (least squares means; n = 8 cannulated cows)

CP Level	LP		HP			*P*-values		
CP Feeding Pattern	OF	SF	OF	SF	SEM	CP level	CP feeding pattern	Interaction
Intake[1]								
DM, kg	25.1	24.3	25.4	25.2	1.1	0.42	0.50	0.71
OM, kg	24.1	23.3	24.2	24.1	1.0	0.48	0.52	0.62
aNDF, kg	7.2	7.0	6.9	6.7	0.3	0.16	0.26	0.93
aNDFom[2], kg	7.0	6.8	6.8	6.6	0.3	0.12	0.25	0.91
pdNDFom[3], kg	5.2	5.0	4.9	4.8	0.2	0.10	0.32	0.61
Apparent digestibility, %								
DM	70.0	68.9	69.8	69.6	1.3	0.84	0.58	0.71
OM	73.2	72.3	73.2	73.1	1.2	0.69	0.61	0.69
aNDF	48.0	45.5	46.4	45.4	2.8	0.73	0.47	0.74
aNDFom	50.5	48.2	48.7	48.6	2.5	0.74	0.56	0.59
pdNDFom	64.6	63.4	65.6	66.4	2.1	0.23	0.90	0.55
CP	69.3	68.9	70.8	70.8	1.3	0.17	0.84	0.86
Output[4]								
Manure, as-is, kg	78.2	75.5	83.8	80.7	4.4	0.08	0.34	0.95
Urine, as-is, kg	25.5	23.4	29.5	28.9	2.2	<0.001	0.20	0.45
Feces, as-is, kg	52.7	52.1	54.2	51.7	3.0	0.79	0.47	0.66
Fecal DM, kg	7.5	7.6	7.7	7.5	0.4	0.74	0.88	0.69

[1]Based on quantity of feed offered and refused during the last 4-d of each 28-d sampling period and chemical composition of feed, feces, and orts samples.

[2]aNDFom = NDF treated with α-amylase and Na_2SO_3 and corrected for ash content.

[3]pdNDFom = potentially digestible NDF, corrected for ash content.

[4]From 4-d total collection and sampling of feces and urine.

Table 2. Milk production, N balance and urea-related measurements of cows fed diets varying in crude protein (CP): low protein (LP = 13.8% CP) or high protein (HP = 15.5% CP), with oscillating (OF, ± 1.8% CP at 48-h intervals) or static (SF) feeding patterns (Least squares means; n = 8 cannulated cows)[1]

CP Level	LP		HP			*P*-values		
CP Feeding Pattern	OF	SF	OF	SF	SEM	CP Level	CP Feeding Pattern	Interaction
Milk, kg/d	39.8	37.9	39.3	38.9	2.0	0.81	0.19	0.37
FPCM, kg/d[2]	37.0	36.6	37.2	37.6	1.5	0.44	0.99	0.66
BW	666	664	673	676	24	0.25	0.93	0.78
BCS	3.14	3.14	3.24	3.16	0.09	0.17	0.44	0.40
Nitrogen, g/d								
Intake	555	534	632	624	26	<0.001	0.43	0.70
Milk	179	174	183	180	8	0.40	0.43	0.85
Urinary	153	155	210	205	8	<0.001	0.82	0.52
Fecal	170	168	186	180	9	0.08	0.58	0.79
Residual	54	37	54	60	16	0.32	0.67	0.33
Nitrogen, % of intake N								
Milk	33.1	32.9	30.1	29.3	1.3	<0.001	0.27	0.41
Urinary	28.6	29.3	34.4	33.2	1.0	<0.001	0.78	0.20
Fecal	30.9	31.5	29.8	29.1	1.3	0.18	0.96	0.62
Manure	59.6	60.8	64.4	62.3	1.8	0.08	0.79	0.34
Residual	7.6	6.2	5.8	8.3	2.4	0.93	0.76	0.27
Urinary components								
Creatinine, mg/L	664	680	582	611	42	<0.01	0.29	0.74
Creatinine, g/d	16.94	16.65	17.55	17.42	0.61	0.02	0.45	0.76
Creatinine, mg/kg BW	25.48	25.23	26.36	25.97	0.71	0.11	0.85	0.88
UUN, g/L	3.55	4.10	4.70	4.91	0.32	<0.001	0.05	0.32
UUNY, g/d	93	98	146	142	6	<0.001	0.87	0.30
PUN, mg/dL	10.9	11.5	14.8	14.6	0.5	<0.001	0.49	0.18
UCR, L/min.	0.60	0.60	0.70	0.69	0.04	<0.01	0.98	0.90

[1]Data collected during the last 4-d of each 28-d sampling period.

[2]FPCM = fat- and protein-corrected milk calculated per IDF (2022) as [(1.226 × milk fat concentration (g/100 g)) + (0.0776 × milk true protein concentration (g/100 g)) + 0.2534)]; BCS = body condition score from a scale in 0.25 increments from 1 (thin) to 5 (obese); PUN = plasma urea nitrogen, UUN = Urine urea-N concentration, UUNY = Urine urea-N yield, UCR = Urea clearance rate.

consistent with literature reviewed and meta-analyzed by Spek et al. (2013b) where renal reabsorption of urea appeared to increase (or glomerular filtration rate decreased) as dietary CP decreased from approximately 17 to 13%. Contrary to our hypothesis, we found no differences in residual N (i.e., apparent N retention) amount or percentage due to dietary treatments (Table 2). In our study, the rate of creatinine excretion per unit BW differed from conventional creatinine coefficients (Valadares et al., 1999), which represents an area for future research. Although greater creatinine excretion with HP could indicate differences in postabsorptive N metabolism due to CP level our crossover trial was not designed to measure changes in BW, BCS, or protein turnover. Liu et al. (2021) recently showed that individual cows' milk production responses to dietary CP were uncorrelated with their responses in empty BW gain. Future long-term trials are needed to investigate the effects of protein nutrition on N partitioning to milk and body pools.

We found no effects of CP feeding pattern on N partitioning to milk, excreta, and residual pools, regardless of the dietary CP level. This is counter to our hypothesis that OF would increase partitioning to milk and body retention and reduce N outputs in manure. Interestingly, we found UUN concentration was lesser with OF than SF (4.13 vs 4.51 g/L, respectively, Table 2). This appeared in contrast with Rauch et al. (2021) and Tebbe and Weiss (2020; tendency) where oscillating CP was associated with greater MUN (Powell et al., 2011). In our trial, UUNY was similar across CP feeding patterns, so lesser UUN concentration with OF was probably related to numerical differences in urine output leading to dilution. Although Kohler (2016) showed that oscillating CP increased residual N, suggesting greater body N retention, few studies have quantified changes in lactating cow body protein reserves in response to CP feeding pattern. Using the urea dilution method, Tebbe and Weiss (2020) showed that changes in body composition across a 50-d treatment period in late lactation did not differ due to CP feeding pattern. Therefore, there is currently no evidence that oscillating CP promotes more desirable N partitioning or lessens the outputs of environmentally-reactive N compounds such as urea compared with static CP feeding of lactating cows.

Nitrogen and Feed Efficiency Metrics

Feed efficiency and NUE were similar across conditions in our trial, except that greater dietary CP decreased NUE (32.1 vs. 28.7%; Table 3, n = 16 cows). More specifically, the HP diets increased N intake yet did not improve milk production which suggests that the additional dietary CP was excreted as manure N or retained in body tissues (Dijkstra et al., 2013; Powell et al., 2011). This is consistent with prior research demonstrating NUE generally decreases with increasing N intake (Reynal and Broderick, 2005; Brito and Broderick, 2006; Spanghero and Kowalski, 2021). Although NUE can be affected by dietary protein digestibility (Broderick et al., 2009), digestion kinetics (Mutsvangwa et al., 2016), and AA composition (Gidlund et al., 2015), our diets were designed to minimize differences in RDP:RUP ratios and the AA composition of MP across treatments. Still, considering that reducing NDF and increasing starch may improve NUE independent of dietary CP (Broderick, 2003) it is plausible that part of the observed improvement in NUE in our trial was attributable to the greater inclusion of fermentable carbohydrate in the LP diets. Similar to our findings, CP feeding pattern did not affect NUE in recent trials (Kohler, 2016; Tebbe and Weiss, 2020b; Rauch et al., 2021), although these trials suggested possible differences in aspects of N metabolism. In our trial, feed efficiencies did not differ across experimental conditions because DMI and milk production were similar across CP levels and CP feeding patterns. Our findings corroborate research showing that increasing CP above requirements did not improve milk production performance (Barros et al., 2017; Broderick et al., 2009), and that various oscillating CP feeding patterns did not alter feed efficiency (Tebbe & Weiss, 2020; Kohler, 2016; Rauch et al., 2021).

Compared with HP-feeding, LP feeding resulted in substantially greater MPY/MUNY (248 versus 328 g/g, respectively, $P < 0.001$; Table 3). Interestingly, although CP feeding pattern did not affect NUE in our trial, OF tended to increase the MPY/MUNY ratio relative to SF (295 vs. 281 g/g, $P = 0.066$; Table 3). Authors suggested MPY/MUNY ratio as a practical indicator of N efficiency (Barros et al., 2017; Chen et al., 2022) because its components are easily measurable and differentiate milk N secretions with economic value (milk true protein) from non-protein N that is irreversibly lost from the animal. With late-lactation cows fed 11.8 to 16.2% CP, Barros et al. (2017) found the minimal MPY/MUNY ratio was 279 and co-occurred with maximal milk true protein yield. These authors suggested that lower MPY/MUNY ratio could reflect excess dietary CP or poor utilization of dietary CP whereas greater MPY/MUNY could indicate dietary CP deficiency. Under the conditions of our study, it appears that MPY/MUNY ratio as high as 328 (average for LP diets) was not associated with adverse milk production or milk composition outcomes (Table 3). The differences in MPY/MUNY require further investigation as they suggest that N metabolism differed

based on CP level and potentially CP feeding pattern. Contrary to our hypothesis, there is no evidence for an interaction.

Temporal Responses in Urinary and Plasma Urea-N

Figure 1 shows the yield (i.e., UUNY) and concentration (i.e., UUN) of urea-N in urine and the urea-N concentration in plasma (PUN) over time. Urinary urea-N yield, UUN, and PUN were consistently greater when cows were fed the HP compared with the LP diets. Although CP feeding pattern did not affect UUN or PUN when averaged across time points as discussed in the previous section, Figure 1 shows significant day by CP feeding pattern interactions where urea-N in plasma and excreta rose and fell in response to changes in dietary CP. For OF, UUNY, UUN, and PUN gradually rose after the start of higher-CP feeding and fell after lower-CP feeding. These patterns are similar to a rise and fall in milk urea-N concentration and yield reported in our earlier paper (Erickson et al., 2023). Most existing research has studied the effects of dietary N on UUN, UUNY, and PUN after adaptation to a time-invariant dietary CP level (e.g., Barros et al., 2019). In contrast, our research examined these relationships after adaptation to a condition with time-varying dietary CP (OF) compared with time-invariant dietary CP (SF). Because these temporal patterns represent the response to dietary CP within cow and period, the rise and fall in OF relative to SF likely isolates differences due to diet, independent of cow and contextual factors (e.g., changes in BW, season, or stage of lactation). Therefore, it is interesting to note that the ranges in UUNY of 38.7 g/8-h for the OF-HP diet and 37.9 g/8-h for the OF-LP diet align with previous meta-analyses of treatment means. Spek et al. (2013a) and Powell et al. (2011) suggested that a 1 percentage unit increase in dietary CP was associated with an increase of 28–32 g UUNY/d, or 9.3 to 10.7 g UUNY/8-h. This implies that the 3.5 percentage units difference in dietary CP from the lower- to higher-CP phases for OF conditions would result in a 35 g/8-h difference in UUNY. Our results demonstrate that the dietary CP to UUNY relationships established with meta-analyses (e.g., Powell et al., 2011; Spek et al., 2013a) may be true not only after long-term (1–3 wk.) adaptation to a given dietary CP level, but also when CP changes at shorter intervals such as the 48-h interval in our trial.

Plasma AA concentrations

Table 4 shows the average AA concentrations in composite plasma samples. Across all treatments in our study, plasma concentrations of Met were low relative to previous reports (Martineau et al., 2019). Concentrations of Met and His were greater with HP diets. Concentrations of Val were greater in when cows were fed the HP compared with the LP diets, leading to greater plasma EAA concentrations and a tendency for greater BCAA concentrations. These differences are likely due to greater metabolizable AA supply in HP diets. By deleting certain AA from duodenal infusions, Haque et al. (2013) showed that the relative supplies of individual BCAA could affect animal performance due to synergistic and antagonistic interactions among BCAA and with other EAA. In contrast, we found that plasma concentrations of other EAA and milk protein production were similar across treatment conditions (production data reported in Erickson et al., 2023). The concentration of Gly was substantially greater when cows were fed the LP compared with the HP diets, which drove a tendency for greater plasma NEAA in the LP condition. Plasma Gly enrichment may result from net mobilization of body protein reserves (Ríus, 2019) or sparing of Gly from endogenous urinary N excretion (e.g., as creatinine, purine derivatives, and

Table 3. N efficiency metrics of cows fed diets varying in crude protein (CP): low protein (LP = 13.8% CP) or high protein (HP = 15.5% CP), with oscillating (OF, ± 1.8% CP at 48-h intervals) or static (SF) feeding patterns (Least squares means; n = 16 cows)[1]

Crude Protein Level	LP		HP			*P*-values		
Feeding Pattern	OF	SF	OF	SF	SEM	CP level	CP feeding pattern	Interaction
NUE[2], g/g	31.9	32.3	29.0	28.4	0.8	<0.001	0.76	0.27
MPY/MUNY[3], g/g	333	322	256	240	9	<0.001	0.07	0.74
Milk yield/DMI, kg/kg	1.55	1.56	1.54	1.54	0.05	0.45	0.66	0.87
FPCM[4]/DMI, kg/kg	1.49	1.53	1.51	1.52	0.04	0.88	0.34	0.71

[1]Data collected during the last 4-d of each 28-d sampling period.

[2]NUE = nitrogen use efficiency; 100 x (milk true protein N / Intake N).

[3]MPY/MUNY = ratio of milk protein yield (g) to milk urea-N yield.

[3]MPY/MUNY = ratio of milk protein yield (g).

[4]FPCM = fat-protein-corrected milk calculated per IDF (2022) as [(1.226 × milk fat concentration (g/100 g)) + (0.0776 × milk true protein concentration (g/100 g)) + 0.2534)].

hippuric acid; Lapierre et al., 2020). In contrast to our findings, Omphalius et al. (2019) recently reported lower dietary protein reduced plasma Gly concentrations and increased mammary NEAA uptake. Therefore, the response of plasma Gly concentration to dietary protein may be mediated by changes in body and mammary tissue metabolism. We observed no effects of the CP feeding pattern except on Pro, which was greater when cows were fed the OF compared with the SF diets, and on Asn, which tended to be greater in the OF diet. Non-essential AA are extensively used by the gastrointestinal tract, so it is plausible that OF induced gross changes or shifted splanchnic metabolism to effect changes in Pro (Lobley et al., 2003). A recent study suggested that oscillating dietary CP increased gastrointestinal tract mass of growing dairy calves (Zhang et al., 2021). However, Tebbe and Weiss (2020) reported that oscillating dietary CP had minimal effects on plasma concentrations of individual AA, the sum of EAA, and the sum of NEAA of lactating cows. These authors also found AA concentrations were similar between higher- and lower-CP oscillation phases (24 h), which corroborates the non-significant feeding pattern by oscillation phase (48 h) effects observed in the present study. Our study is unique from previous reports because we observed that plasma AA differences caused by a 24-d adaptation to a 3.3 percentage units difference in average dietary CP (LP = 13.8, HP = 15.5% CP of DM) were not replicated in the transient response to a 3.3 percentage units difference in dietary CP induced across oscillation phases (OF-LP 12.2–15.5%, OF-HP 13.8–17.3% CP).

Gas Emissions

Table 5 shows gas production, intensity, and yield for the non-cannulated subset of cows in our trial. Results suggested minimal differences in gas emissions related to CP level and CP feeding pattern. Emissions of CH_4 and CO_2 in our trial were comparable to recent reports with similar dietary and cow conditions (Lage et al., 2021). Our results are consistent with Müller et al. (2021) which reported no differences in production of CH_4 and CO_2 and oxygen consumption when lowering protein and increasing starch (13.8% CP) relative to a higher-CP diet (15.9% CP), and Niu et al. (2016) which found no differences in enteric CH_4 production, intensity, or yield when comparing 15.2 and 18.5% dietary CP in a crossover study. In contrast, Gidlund et al. (2015) reported reduced CH_4 yield at moderate dietary CP (18.4–19.1%) compared with higher and lower dietary CP extremes (17.0–17.3% and 20.1–21.0%) where dietary NDF and pdNDF were similar across conditions. Importantly, dietary NDF and pdNDF changed modestly between LP and HP diets in our trial. The absence of CP level and CP feeding pattern effects suggests that conditions for methanogenesis (e.g., substrate availability, microbial community structure and fermentation activity) were similar among conditions.

Interestingly, we found that HP diets, especially OF-HP, tended to promote greater CO_2 production. In ruminants, enteric CH_4 production is largely determined by rumen fermentation capacity and fermentation patterns, whereas CO_2 arises predominantly from respiration and to a lesser extent from fermentation (Madsen et al., 2010). It is unclear whether CO_2 differences were caused at the rumen-level (e.g., alterations to microbial biomass, species composition, or substrate usage), or at the whole-animal level (e.g., changes in macronutrient metabolism, body composition, and maintenance requirements). In a Ussing chamber experiment with ruminal epithelia from growing lambs fed oscillating and static CP diets, Doranelli et al. (2011) found greater urea flux from tissues collected after the lower CP oscillation phase relative to the higher CP phase. Although these authors showed no differences in urea flux between oscillating and static dietary CP treatments, it is possible that differences in urea flux and microbial ureolytic activity could affect intraruminal CO_2 production (Doranelli et al., 2011). At the whole-animal level, Liu et al. (1995) observed that some growing lambs exhibited marked declines in protein synthesis and degradation at 2 d after switching from maintenance to low protein intragastric infusions. Talal et al. (2020) suggested that greater CO_2 yield could occur with lipogenesis or upregulation of the pentose phosphate pathway. Tissue protein turnover could consume oxygen and produce CO_2 without affecting CH_4 production (Hanigan et al., 2009). Additionally, CO_2 emissions could be affected by bicarbonate usage in body buffering systems which HP diets may have affected by increasing urea transport into the rumen and into urine (Laporte-Uribe, 2019; Tebbe and Weiss, 2020). Tracking fermentative and respiratory emissions may increase in importance with carbon measurement and accounting schemes.

CONCLUSION

Our trial tested the effects of CP level, CP feeding pattern, and their interaction on N balance, nutrient digestibility, and gas emissions with mid- to late-lactation Holstein cows. Contrary to our hypothesis, CP feeding pattern had minimal effects on measured variables, regardless of the average CP level (LP = 13.8 vs. HP = 15.5% of DM). Crude protein level did not alter milk N. Instead, feeding the HP diets led to effects consistent with N overfeeding, such as increased

UUNY, UUN, and UCR, and reduced NUE. Time series analysis of UUNY, UUN, and PUN showed that these body and excreta urea-N pools responded to increased and decreased dietary CP within 48-h intervals (2 feedings) and that responses to dietary N were consistent with previous meta-analytical research. Interestingly,

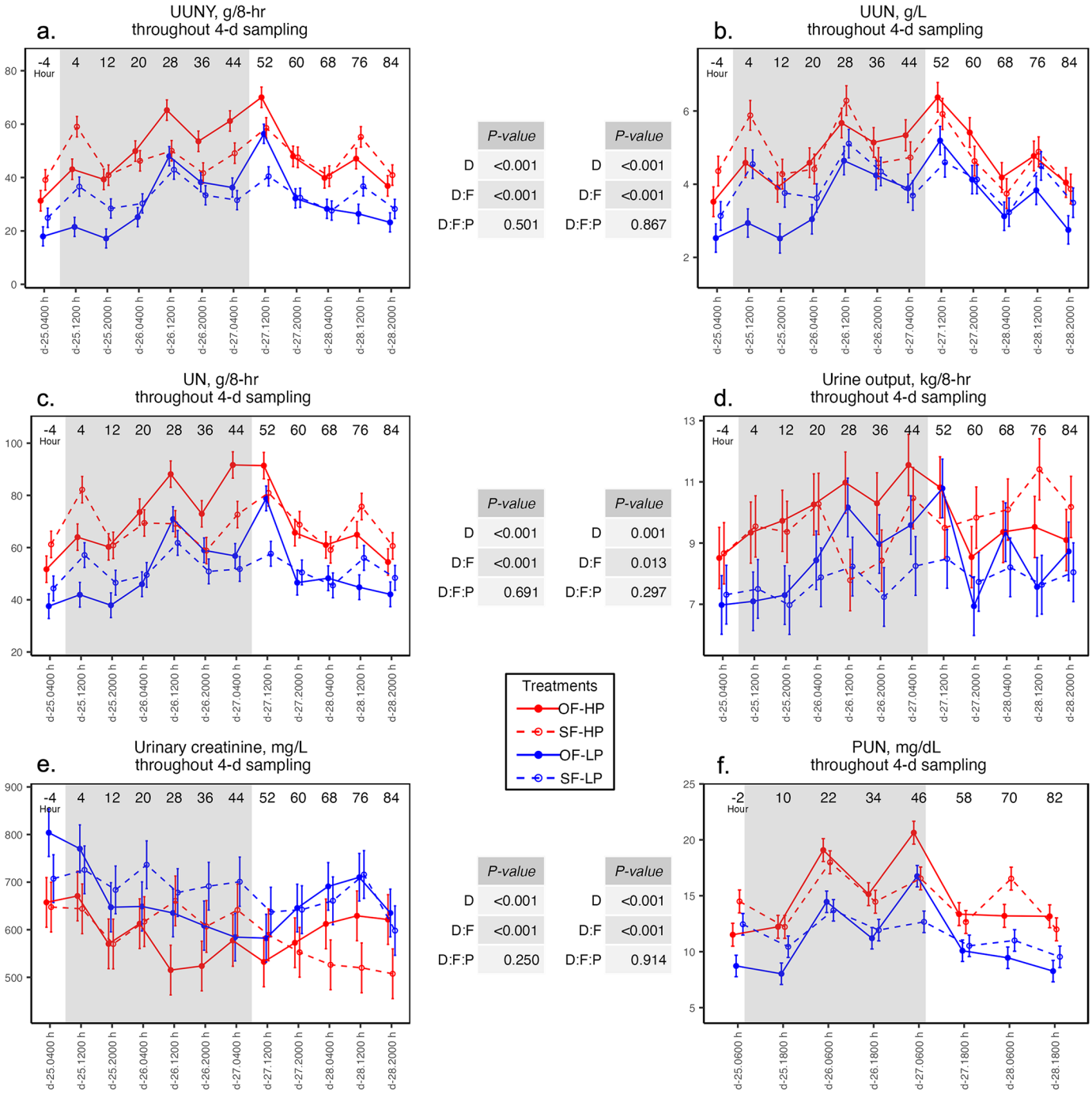

Figure 1. Temporal patterns in variables with significant feeding pattern by day interactions: a) Urinary urea-N yield (UUNY, g/8-h), b) urinary urea-N concentration (UUN, g/L), c) urinary N (g/8-h), d) urine output (kg/8-h), e) urine creatinine (mg/L), and f) plasma urea-N concentration (PUN, mg/dL). Results of F-tests for day (D) by feeding pattern (F) and crude protein level (P) are shown in tables. A gray rectangle shows the higher-CP phase in OF. Points show least squares means from linear mixed models with F, P, F:P, D, and sampling hour (H), with all possible interactions between temporal and treatment variables. Plots represent n = 360 (UUNY, UUN) and n = 240 (PUN) observations from n = 8 cows in the cannulated subset, across a 4-d sampling period under conditions of oscillating high protein (OF-HP, 15.5 ± 1.8% crude protein), and oscillating low protein (OF-LP, 13.8 ± 1.8% crude protein).

the HP diets increased urinary creatinine excretion, increased plasma EAA concentrations, and tended to decrease plasma NEAA concentrations, which could suggest differences in macronutrient metabolism. Production of CO_2 was also greater for cows fed the OF-HP diet compared with the other dietary treatments. In summary, the 48-h dietary CP oscillations in our trial had minimal effects on N balance, nutrient digestibility, and gas fluxes at CP levels near (HP) or below (LP) predicted MP requirements.

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Table 4. AA concentrations in plasma composite samples (n = 64) of cows fed diets varying in crude protein (CP): low protein (LP = 13.8% CP) or high protein (HP = 15.5% CP), with oscillating (OF, ± 1.8% CP at 48-h intervals) or static (SF) feeding patterns (Least squares means; n = 8 cannulated cows)[1]

CP Level	LP		HP			*P*-values			
CP Feeding Pattern	OF	SF	OF	SF	SEM	CP Level	CP Feeding Pattern	CP Level * CP Feeding Pattern	CP Feeding Pattern * Oscillation phase[3]
AA[2], μM									
Arg	63.4	64.5	70.6	66.4	3.4	0.06	0.53	0.28	0.62
His	47.2	45.0	49.9	49.1	1.9	0.04	0.38	0.67	0.60
Ile	158.4	160.1	166.7	158.5	8.8	0.56	0.59	0.40	0.17
Leu	164.2	170.7	178.0	171.3	8.9	0.27	0.99	0.31	0.33
Lys	83.3	86.0	85.1	87.2	4.6	0.69	0.54	0.93	0.78
Met	15.1	14.9	16.8	16.1	0.8	0.03	0.48	0.67	0.42
Phe	52.1	49.9	62.6	52.3	4.4	0.11	0.12	0.31	0.05
Thr	93.9	93.4	94.2	92.8	5.1	0.94	0.74	0.87	0.49
Trp	52.6	49.7	50.8	50.8	2.5	0.80	0.32	0.30	0.31
Val	265.6	268.9	294.3	290.4	13.0	<0.01	0.97	0.62	0.25
Ala	278.7	265.0	271.5	264.5	14.8	0.73	0.37	0.77	0.96
Asn	28.9	25.5	26.9	25.1	2.2	0.42	0.08	0.59	0.97
Asp	13.3	11.1	14.9	13.3	2.0	0.30	0.30	0.86	0.28
Gln	180.3	171.2	191.1	178.9	8.5	0.22	0.17	0.84	0.61
Glu	110.1	105.9	111.5	107.3	4.3	0.60	0.15	0.99	0.58
Gly	374.7	344.8	284.2	297.7	23.5	<0.01	0.63	0.19	0.25
Pro	92.7	88.7	95.1	87.4	4.7	0.82	0.02	0.44	0.87
Ser	101.0	101.1	105.3	93.9	5.5	0.76	0.24	0.22	0.70
Tyr	52.5	53.0	58.7	55.3	3.0	0.08	0.55	0.41	0.07
TAA	2227	2175	2230	2164	61	0.93	0.20	0.87	0.16
EAA	995	1006	1069	1037	40	0.04	0.69	0.40	0.18
NEAA	1230	1170	1159	1127	37	0.07	0.15	0.65	0.38
BCAA	588	600	639	620	29	0.05	0.87	0.40	0.21

[1]Data collected during the last 4-d of each 28-d sampling period.

[2]TAA = total AA concentration (μM); EAA = essential AA (μM); NEAA = non-essential AA (μM); BCAA = branched chain AA (μM).

[3]The CP feeding pattern by oscillation phase interaction shows whether the effects of 48-h phase on the analyte differed for static CP feeding versus oscillating CP feeding. A significant interaction indicates a different temporal pattern of response with OF versus SF across two 48 h phases in d 25 to d 28 of an experimental period.

Table 5. Gas emissions, oxygen consumption, and respiratory quotient cows fed diets varying in crude protein (CP): low protein (LP = 13.8% CP) or high protein (HP = 15.5% CP), with oscillating (OF, ± 1.8% CP at 48-h intervals) or static (SF) feeding patterns (Least squares means; n = 8 non-cannulated cows)[1]

CP Level	LP		HP			*P*-values		
CP Feeding Pattern	OF	SF	OF	SF	SEM	CP level	CP feeding pattern	Interaction
DMI, kg/d	26.4	26.8	26.4	25.9	1.0	0.40	0.94	0.44
Milk, kg/d	37.0	36.7	37.2	35.9	1.5	0.73	0.35	0.60
FPCM[2], kg/d	37.5	36.3	37.2	36.2	1.6	0.82	0.27	0.93
BW	657	660	669	662	21	0.46	0.85	0.59
BCS	3.14	3.14	3.19	3.16	0.11	0.49	0.79	0.71
CH_4								
Production, g/d	473	473	483	466	26	0.89	0.48	0.46
Intensity, g/kg FPCM	12.7	13.0	13.2	13.0	0.7	0.59	0.92	0.54
Yield, g/kg DMI	17.9	17.5	18.5	18.1	0.8	0.28	0.49	0.92
CO_2								
Production, g/d	14,830	14,870	15,600	14,890	410	0.07	0.12	0.08
Intensity, g/kg FPCM	402	417	427	419	19	0.23	0.73	0.33
Yield, g/kg DMI	564	557	595	586	16	0.02	0.47	0.93
CH_4/CO_2, L/L	0.964	0.950	0.931	0.943	0.004	0.31	0.95	0.50
Oxygen consumption, g/d	10,200	10,220	10,600	10,420	332	0.10	0.65	0.57
Respiratory quotient[3]	1.05	1.05	1.07	1.03	0.02	0.87	0.27	0.26

[1]Data collected during the last 4-d of each 28-d sampling period.

[2]FPCM = fat-protein-corrected milk calculated per IDF (2022) as [(1.226 × milk fat concentration (g/100 g)) + (0.0776 × milk true protein concentration (g/100 g)) + 0.2534)].

[3]Respiratory quotient = CO_2 (L/d) of CO_2 emitted divided by O_2 consumed (L/d).

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APPENDIX

Appendix Table 1. Target and predicted efficiencies of metabolizable AA use for cows (n = 16) fed diets varying in crude protein (CP): low protein (LP = 13.8% CP) or high protein (HP = 15.5% CP), with oscillating (OF, ± 1.8% CP at 48-h intervals) or static (SF) feeding patterns[1]

AA	Target	OF-LP Low phase	OF-HP Low phase; SF-LP	OF-LP High phase; SF-HP	OF-HP High phase
Arg		0.59	0.51	0.44	0.40
His	0.75	1.00	0.87	0.78	0.70
Ile	0.71	0.77	0.68	0.61	0.55
Leu	0.73	0.88	0.79	0.71	0.65
Lys	0.72	0.87	0.76	0.68	0.61
Met	0.73	1.01	0.90	0.81	0.74
Phe	0.60	0.71	0.62	0.56	0.51
Thr	0.64	0.76	0.67	0.60	0.55
Trp	0.86	0.98	0.86	0.76	0.68
Val	0.74	0.87	0.77	0.69	0.63

[1]Based on NASEM (2021) using measured DMI, milk yield and composition, BW, DIM, and days in gestation for the trial averaged across all cows.