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Effects of protein supplementation frequency on physiological responses associated with reproduction in beef cows¹

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ABSTRACT: The objective of this experiment was to determine if frequency of protein supplementation impacts physiological responses associated with reproduction in beef cows. Fourteen nonpregnant, nonlactating beef cows were ranked by age and BW and allocated to 3 groups. Groups were assigned to a 3 × 3 Latin square design, containing 3 periods of 21 d and the following treatments: 1) soybean meal supplementation daily (D), 2) soybean meal supplementation 3 times/week (3WK), and 3) soybean meal supplementation once/week (1WK). Within each period, cows were assigned to an estrus synchronization protocol: 100 µg of GnRH + controlled internal drug release device (CIDR) containing 1.38 g of progesterone (P₄) on d 1, 25 mg of PGF_{2α} on d 8, and CIDR removal + 100 µg of GnRH on d 11. Grass-seed straw was offered for ad libitum consumption. Soybean meal was individually supplemented at a daily rate of 1 kg/cow (as-fed basis). Moreover, 3WK was supplemented on d 0, 2, 4, 7, 9, 11, 14, 16, and 18 whereas 1WK was supplemented on d 4, 11, and 18. Blood samples were collected from 0 (before) to 72 h after supplementation on d 11 and 18 and analyzed for plasma urea-N (PUN). Samples collected from 0 to 12 h were also analyzed for plasma glucose,

insulin, and P₄ (d 18 only). Uterine flushing fluid was collected concurrently with blood sampling at 28 h for pH evaluation. Liver biopsies were performed concurrently with blood sampling at 0, 4, and 28 h and analyzed for mRNA expression of carbamoyl phosphate synthetase I (CPS-I; h 28) and CYP2C19 and CYP3A4 (h 0 and 4 on d 18). Plasma urea-N concentrations were greater ($P < 0.01$) for 1WK vs. 3WK from 20 to 72 h and greater ($P < 0.01$) for 1WK vs. D from 16 to 48 h and at 72 h after supplementation (treatment × hour interaction, $P < 0.01$). Moreover, PUN concentrations peaked at 28 h after supplementation for 3WK and 1WK ($P < 0.01$) and were greater ($P < 0.01$) at this time for 1WK vs. 3WK and D and for 3WK vs. D. Expression of CPS-I was greater ($P < 0.01$) for 1WK vs. D and 3WK. Uterine flushing pH tended ($P \leq 0.10$) to be greater for 1WK vs. 3WK and D. No treatment effects were detected ($P \geq 0.15$) on expression of CYP2C19 and CYP3A4, plasma glucose, and P₄ concentrations, whereas plasma insulin concentrations were greater ($P \leq 0.03$) in D and 3WK vs. 1WK. Hence, decreasing frequency of protein supplementation did not reduce uterine flushing pH or plasma P₄ concentrations, which are known to impact reproduction in beef cows.

Key words: beef cows, plasma urea-N, progesterone, protein, supplementation frequency, uterine pH

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INTRODUCTION

Fall-calving herds often require protein supplementation during the winter breeding season, particularly due to the limited availability and nutritional content of available forages (DelCurto et al., 2000). Nevertheless, supplementation programs substantial-

ly increase production costs in cattle systems, including expenses associated with feed purchase and labor required for supplement feeding (Miller et al., 2001). Hence, offering supplements 3 times or once weekly instead of daily are common strategies to lessen supplementation and overall production costs in cow-calf operations.

Research has shown that pregnant cattle consuming low-quality forages and receiving protein supplementation as infrequent as once a week had similar BW and BCS compared with cohorts supplemented daily (Huston et al., 1999b). However, no research has yet evaluated the effects of infrequent protein supplementation to beef cows during the breeding season. Decreasing protein supplementation frequency results in increased protein intake per meal as well as the size of each meal. Excessive protein intake increases plasma urea-N (**PUN**) concentrations to levels that may decrease uterine pH (Elrod and Butler, 1993), which impairs reproductive function of beef females by reducing sperm motility and embryo survival (Acott and Carr, 1984; Butler, 1998). Large meal size may reduce circulating progesterone (**P₄**) concentrations, the hormone required for establishment and maintenance of pregnancy (Spencer and Bazer, 2002), by stimulating hepatic blood flow and subsequent hepatic **P₄** catabolism (Sangsritavong et al., 2002). Based on this rationale, we hypothesized that beef cows infrequently supplemented would have reduced uterine pH and circulating **P₄** concentrations following a supplementation event. Hence, our objective was to determine the effects of protein supplementation frequency on physiological responses and hepatic expression of genes associated with metabolism and reproductive function of beef cows.

MATERIALS AND METHODS

This experiment was conducted at the Oregon State University – Eastern Oregon Agricultural Research Center (Burns Station) from October 2013 to January 2014. All animals used herein were cared for in accordance with acceptable practices and experimental protocols reviewed and approved by the Oregon State University Institutional Animal Care and Use Committee (number 4510).

Animals and Diets

Fourteen nonpregnant, nonlactating mature Angus × Hereford cows (initial BW = 471 ± 13 kg and age 4.1 ± 0.4 yr) were ranked by initial BW and age and allocated to 3 groups (2 groups containing 5 cows and 1 group containing 4 cows). Groups were assigned to a 3 × 3 Latin square design, containing 3 periods of 21 d each and the following treatments: 1) soybean meal supplementation daily (**D**), 2) soybean

meal supplementation 3 times/week (**3WK**), and 3) soybean meal supplementation once/week (**1WK**). Within each period (d 1 to 21), cows were assigned to the following estrus synchronization protocol: 100 µg of GnRH (Factrel; Zoetis, Florham Park, NJ) + controlled internal drug release device (**CIDR**) containing 1.38 g of **P₄** (Zoetis) on d 1 and 25 mg of PGF2α (Lutalyse; Zoetis) on d 8 followed by CIDR removal + 100 µg of GnRH on d 11 of each period. The CIDR was maintained in cows for an additional 72 h compared with the original Co-Synch + CIDR protocol (Lamb et al., 2001) to prevent cows from entering estrus before the beginning of sample collection, given that estrus alters uterine pH in beef females (Perry and Perry, 2008a,b).

All animals had ad libitum access to grass-seed straw during the entire experiment. Soybean meal was individually supplemented to cows (at 0700 h) at a daily rate of 1 kg/cow (as-fed basis). Cows receiving 3WK were supplemented on Mondays, Wednesdays, and Fridays (d 0, 2, 4, 7, 9, 11, 14, 16, and 18 of each period; 2.33 kg of soybean meal/feeding), whereas 1WK was supplemented on Fridays (d 4, 11, and 18 of each period; 7 kg of soybean meal/feeding), in such a manner that all cows received the same amount of supplement on a weekly basis. Cows from all treatments consumed their entire supplement allocation within 30 min after feeding. Water and a complete commercial mineral–vitamin mix (Cattleman's Choice; Performix Nutrition Systems, Nampa, ID), containing 14% Ca, 10% P, 16% NaCl, 1.5% Mg, 3,200 mg/kg of Cu, 65 mg/kg of I, 900 mg/kg of Mn, 140 mg/kg of Se, 6,000 mg/kg of Zn, 136,000 IU/kg of vitamin A, 13,000 IU/kg of vitamin D₃, and 50 IU/kg of vitamin E, were offered for ad libitum consumption throughout the experiment.

A sample of straw and soybean meal was collected before the beginning of the experiment and analyzed for nutrient content by a commercial laboratory (Dairy One Forage Laboratory, Ithaca, NY). Samples were analyzed in triplicates by wet chemistry procedures for concentrations of CP (method 984.13; AOAC, 2006), ADF (method 973.18 modified for use in an Ankom 200 fiber analyzer; Ankom Technology Corp., Fairport, NY; AOAC, 2006), and NDF (Van Soest et al., 1991; modified for use in an Ankom 200 fiber analyzer; Ankom Technology Corp.). Calculations for TDN used the equations proposed by Weiss et al. (1992), whereas NEm and NEg were calculated with the equations proposed by the NRC (1996). Nutritive values for straw and soybean meal were, respectively (DM basis), 54 and 80% TDN, 73 and 11% NDF, 50 and 8% ADF, 0.97 and 1.93 Mcal/kg of NEm, 0.42 and 1.27 Mcal/kg of NEg, and 4.7 and 54.1% CP.

Sampling

Individual full BW was recorded at the beginning (d 1) and at the end (d 21) of each experimental period to evaluate if cows were in adequate nutritional status during the experiment, which is known to impact the reproductive function in beef females (Randel, 1990; Bossis et al., 2000; Hess et al., 2005).

Blood Samples. Blood samples were collected from 0 (immediately before) to 72 h after supplements were offered on d 11 and 18 of each period. More specifically, samples were collected every 2 h from 0 to 12 h, every 4 h from 16 to 28 h, and every 12 h from 36 to 72 h after supplementation. This sampling schedule was adopted to assess the impacts of infrequent protein supplementation on 1) d 11, when cows would be inseminated following the estrus synchronization protocol, given that uterine pH impacts sperm motility and viability (Acott and Carr, 1984), and 2) d 18, corresponding to d 7 of gestation, when uterine pH and circulating P_4 concentrations are known to modulate embryonic development and survival (Mann and Lamming, 2001; Ocón and Hansen, 2003).

Blood samples were collected via jugular venipuncture into commercial blood collection tubes (Vacutainer, 10 mL; Becton Dickinson, Franklin Lakes, NJ) containing 158 United States Pharmacopeia units of freeze-dried sodium heparin. After collection, blood samples were immediately placed on ice and centrifuged ($2,500 \times g$ for 30 min at 4°C) for plasma harvest, and plasma was stored at -80°C on the same day of collection. Blood samples collected from 0 to 72 h were analyzed for PUN concentrations, while samples collected from 0 to 12 h were analyzed for plasma glucose, insulin, and P_4 concentrations (d 18 only). Additionally, blood samples collected 24 h after supplement feeding on d 11 were also analyzed for plasma P_4 concentrations, to assess estrus synchronization rate. A cow was considered responsive to the estrus synchronization protocol if plasma P_4 concentration was <1.0 ng/mL on the aforementioned sample and >1.0 ng/mL on the first sample collected on d 18. Plasma PUN and glucose concentrations were determined using quantitative colorimetric kits (number B7551 and G7521, respectively; Pointe Scientific, Inc., Canton, MI). Plasma insulin and P_4 concentrations were analyzed using a chemiluminescent enzyme immunoassay (Immulite 1000; Siemens Medical Solutions Diagnostics, Los Angeles, CA). Only cows that responded to the estrus synchronization protocol had all samples from d 18 analyzed for plasma P_4 concentrations. The intra- and interassay CV were, respectively, 3.78 and 11.05% for PUN and 3.78 and 6.44% for glucose. All samples were analyzed for insulin and P_4 concentrations within a single assay, and the intra-assay CV were 2.3 and 3.8%, respectively.

Uterine Flushing. One month before the beginning of the experiment, a pretrial with 9 nonlactating and nonpregnant Angus \times Hereford cows assigned to the same treatments (3 cows/treatment) and blood sampling schedule reported herein was conducted to evaluate when PUN concentrations would peak following supplementation. These results determined the appropriate sampling time for uterine flushing, more specifically when PUN peaked due to the negative correlation among PUN and uterine pH reported by Hammon et al. (2005). In this pretrial, PUN concentrations were numerically stable for D and peaked at 28 h after 3WK and 1WK (20.26, 28.68, and 32.72 mg/dL, respectively, at 28 h relative to supplementation; SEM = 1.93). Hence, in the present experiment, uterine flushing fluid was collected 28 h after supplementation on d 11 and 18 for pH measurement according to procedures previously described by Hersom et al. (2010). More specifically, uterine flush samples were collected by passing a sterile Foley 2-way, 16-French catheter (C. R. Bard, Covington, GA) into the uterus. Thirty-five milliliters of sterile saline (0.9%; pH = 7.0) was gently infused through the catheter. Saline was allowed to equilibrate for 90 s and then flushed from the uterus through the catheter into a 50-mL Falcon tube (BD Biosciences, Bedford, MA). Flushing fluid was measured for pH immediately after collection (Orion SA 520; American Instrument Exchange Inc., Haverhill, MA). The pH of sterile saline was used to standardize the pH calibration before each cow was flushed.

Liver Samples. Liver sampling was performed via needle (Tru-Cut biopsy needle; CareFusion Corporation, San Diego, CA) biopsy 28 h after supplement feeding on d 11 and at 0, 4, and 28 h relative to supplement feeding on d 18 according to the procedures described by Arthington and Corah (1995). During biopsies, incisions were made between the 11th and 12th ribs for collection of samples from the right hepatic lobe (Miranda et al., 2010) and at least 2 cm from previous incision or incisions of the same period to prevent collection of damaged hepatic tissue. Immediately after collection, liver samples (average 100 mg of tissue; wet weight) were placed in 1 mL of RNA stabilization solution (RNAlater; Ambion Inc., Austin, TX), maintained at 4°C for 24 h, and stored at -80°C . Samples were analyzed via real-time quantitative reverse transcription (RT)-PCR for carbamoyl phosphate synthetase I (CPS-I; rate-limiting enzyme in the urea cycle; Takagi et al., 2008), CYP2C19 and CYP3A4 (enzymes that regulate P_4 steroid catabolism; Lemley et al., 2008), and ribosomal protein S-9 (RPS-9; hepatic housekeeping gene; Janovick-Guretzky et al., 2007) mRNA expression. Expression of CPS-I was assessed only on samples collected 28 h relative to supplementation based on the results from our pretrial.

Expression of CYP2C19 and CYP3A4 was assessed in samples collected at 0 and 4 h relative to supplementation on d 18 from cows that responded to the estrus synchronization protocol, based on previous research from our group (Vieira et al., 2013).

Total RNA was extracted from liver tissue samples using the TRIzol Plus RNA Purification Kit (Invitrogen, Carlsbad, CA) and stored at -80°C until further processing. Quantity and quality of isolated RNA were assessed via UV absorbance (NanoPhotometer Version 2.1; Implen, Munich, Germany) at 260 nm and 260:280 nm ratio, respectively (Fleige and Pfaffl, 2006). Extracted RNA (2.5 μg) was incubated at 37°C for 30 min in the presence of RNase-free (**DNase**; New England Biolabs Inc., Ipswich, MA) to remove contaminant genomic DNA. After inactivating the DNase (75°C for 15 min), samples were reverse transcribed using the High Capacity cDNA Reverse Transcription Kit with random hexamers (Applied Biosystems, Foster City, CA). Real-time RT-PCR was completed using the SYBR Green PCR Master Mix (Applied Biosystems) and specific primer sets (20 pM; Table 1) with a 7900HT Fast Real-time PCR cycler (Applied Biosystems) according to procedures described by Cooke et al. (2008). At the end of each RT-PCR, amplified products were subjected to a dissociation gradient (95°C for 15 s, 60°C for 30 s, and 95°C for 15 s) to verify the amplification of a single product by denaturation at the anticipated temperature. Responses were quantified based on the threshold cycle (CT), the number of PCR cycles required for target amplification to reach a predetermined threshold. All CT responses from genes of interest were normalized to RPS-9 CT examined in the same sample and assessed at the same time as the targets. Results are expressed as relative fold change ($2^{-\Delta\Delta\text{CT}}$), as described by Ocón-Grove et al. (2008).

Statistical Analysis

All data were analyzed using cow as the experimental unit and cow and group as random variables and using the Satterthwaite approximation to determine the denominator degrees of freedom for the tests of fixed effects. Response to estrus synchronization was analyzed using the GLIMMIX procedure of SAS (version 9.3; SAS Inst., Cary, NC), with a model statement containing the effects of treatment and period as independent variables. All other data were analyzed using the MIXED procedure of SAS. The model statement for BW change contained the effects of treatment and period as independent variables. The model statement for plasma variables contained the effects of treatment, day (for PUN, glucose, and insulin), hour, all the resultant interactions, and period as an independent variable. The specified term for the repeated

Table 1. Primer sequences and accession number for all gene transcripts analyzed by quantitative real-time reverse transcription PCR

Target gene	Primer sequence ¹	Accession no.
Carbamoyl phosphate synthetase-I		
Forward	5'-ACACTGGCTGCAGAATACCC-3'	XM_587645
Reverse	5'-TTCTTGCCAAGCTGACGCAA-3'	
CYP2C19		
Forward	5'-TATGGACTCCTGCTCCTGCT-3'	NM_001109792
Reverse	5'-CATCTGTGTAGGGCATGCAG-3'	
CYP3A4		
Forward	5'-GTGCCAATCTCTGTGCTTCA-3'	BT030557
Reverse	5'-CCAGTTCCAAAAGGCAGGTA-3'	
Ribosomal protein S-9		
Forward	5'-CCTCGACCAAGAGCTGAAG-3'	DT860044
Reverse	5'-CCTCCAGACCTCACGTTTGTC-3'	

¹Primer sequences for carbamoyl phosphate synthetase obtained from Takagi et al. (2008), primer sequences for CYP2C19 and CYP3A4 obtained from Lemley et al. (2008), and primer sequences for ribosomal protein S-9 obtained from Janovick-Guretzky et al. (2007).

statement was hour, the subject was cow(treatment \times day \times period) or cow(treatment \times period) for P_4 analysis only, and the covariance structure used was compound symmetry based on the Akaike information criterion. The model statement used for uterine flushing pH contained the effects of treatment, day, synchronization status, all interactions, and period as an independent variable. The model statement for hepatic gene expression contained the effects of treatment, day (for CPS-I) or hour (for CYP2C19 and CYP3A4), all resultant interactions, and period as an independent variable. For uterine flushing pH and CPS-I expression, the specified term for the repeated statement was day, the subject was cow(treatment \times period), and the covariance structure used was first-order autoregressive based on the Akaike information criterion. For CYP2C19 and CYP3A4 expression, the specified term for the repeated statement was hour, subject was cow(treatment \times period), and the covariance structure used was also first-order autoregressive. Results are reported as least-squares means, which were separated using the PDIFF option in SAS. Significance was set at $P \leq 0.05$ and tendencies were denoted if $P > 0.05$ and $P \leq 0.10$. Results are reported according to main effects if no interactions were significant or according to highest-order interaction detected.

RESULTS AND DISCUSSION

No treatment ($P = 0.97$) effects were observed on estrus synchronization rate (79, 80, and 78% for D, 3WK, and 1WK, respectively; SEM = 1.2). Furthermore, BW change did not differ ($P = 0.35$) between treatments (13.4, 18.1, and 13.6 kg of BW change for D, 3WK,

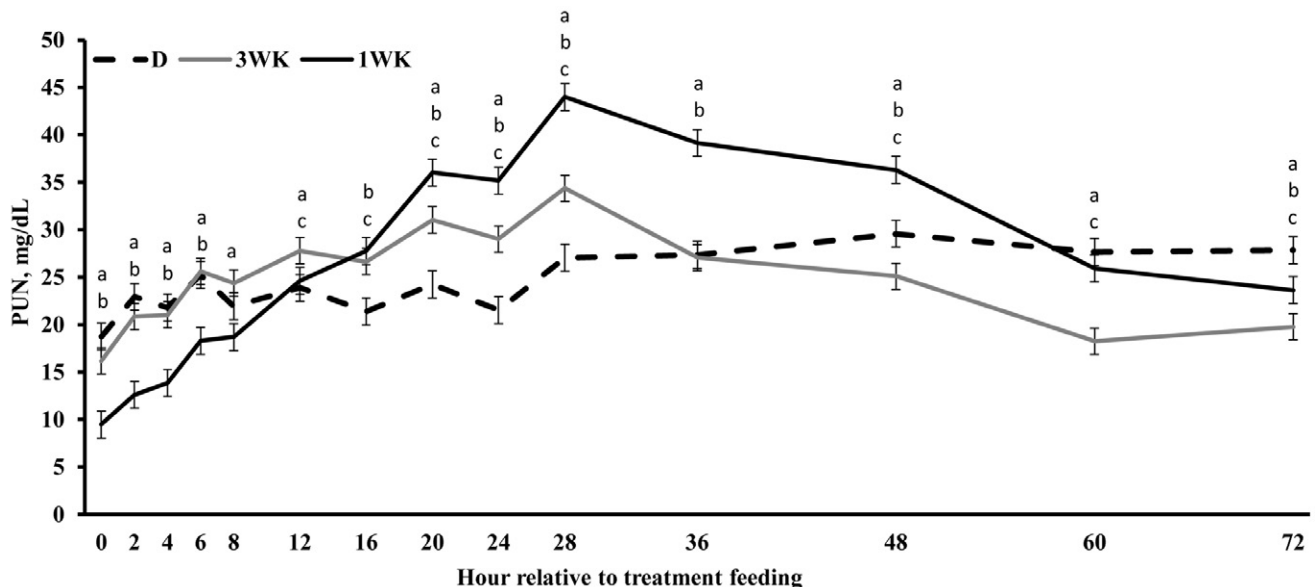


Figure 1. Plasma urea-N (PUN) concentrations in beef cows receiving soybean meal supplementation daily (D; $n = 14$), soybean meal supplementation 3 times/week (3WK; $n = 14$), or soybean meal supplementation once/week (1WK; $n = 14$). Soybean meal was individually supplemented at a daily rate of 1 kg/cow (as-fed basis). Blood samples were collected from 0 (before) to 72 h after all treatments received supplementation. A treatment \times hour interaction was detected ($P < 0.01$). Within hours, letters indicate the following treatment differences: a = 3WK vs. 1WK ($P \leq 0.04$), b = D vs. 1WK ($P < 0.01$), and c = D vs. 3WK ($P \leq 0.01$).

and 1WK, respectively; SEM = 3.0). Although the present experiment was not designed to evaluate these parameters, it is important to note that cows used herein were in similar and positive nutritional status, and responsiveness to the estrus synchronization protocol was adequate to properly test our hypothesis.

Parameters Associated with Protein Intake

A treatment \times hour interaction was detected ($P < 0.01$) for PUN concentrations, which were greater ($P < 0.01$) for 1WK compared with 3WK from 20 to 72 h and greater ($P < 0.01$) for 1WK compared with D from 16 to 48 h and at 72 h after supplementation (Fig. 1). Conversely, D and 3WK had greater ($P < 0.01$) PUN concentrations compared with 1WK from 0 to 6 h relative to supplementation. Concentrations of PUN in 3WK were also greater ($P < 0.01$) from 12 to 28 h and reduced ($P < 0.01$) from 48 to 72 h after supplementation compared with D (Fig. 1). Moreover, PUN concentrations peaked at 28 h after 3WK and 1WK ($P < 0.01$) and were greater ($P < 0.01$) at this time for 1WK and 3WK compared with D as well as for 1WK compared with 3WK (Fig. 1). Similarly, others have also reported that PUN peaked 1 d after supplementation in ruminants consuming low-quality forage and receiving a protein supplement as infrequent as once/week (Krehbiel et al., 1998; Huston et al., 1999a; Bohnert et al., 2002). Treatment effects detected for PUN herein reflect the designed differences in protein intake across treatments for each supplementation event, given that PUN concentrations

are positively correlated with the amount of protein and RDP consumed by ruminants (Broderick and Clayton, 1997; Cappelozza et al., 2014a,b). Moreover, Hammond (1997) suggested that PUN concentrations should range from 7 to 8 mg/dL for mature beef cows. Therefore, cows from all treatments had PUN concentrations as well as N supply (Bach et al., 2005) beyond the adequate range throughout the sampling period.

A treatment effect was detected ($P < 0.01$) for the hepatic mRNA expression of CPS-I on samples collected 28 h after supplements were offered. Cows fed 1WK had greater ($P < 0.01$) hepatic CPS-I mRNA expression compared with cows fed D and 3WK, while CPS-I expression was similar ($P = 0.67$) between cows fed D and 3WK (Table 2). Carbamoyl phosphate synthetase I is expressed in hepatocytes and epithelial cells of the intestinal mucosa (Tillman et al., 1996) and is considered a rate-limiting step within the urea cycle (Takagi et al., 2008) by converting ammonia into carbamoyl phosphate (Vissek, 1979). In addition, expression and activity of urea cycle enzymes are affected by hormones and nutrients (Takiguchi and Mori, 1995). Hence, the greater mRNA expression of CPS-I in 1WK compared with 3WK and D corroborate treatment differences in protein intake as well as PUN concentrations 28 h after supplementation (Ryall et al., 1984; Hayden and Straus, 1995; Takagi et al., 2008). Nevertheless, the similar CPS-I mRNA expression between 3WK and D was unexpected, although others have also reported that enzyme activity can be increased without changes in its expression (Banu et al., 2009; Lebovic et al., 2013).

Table 2. Uterine flushing pH and expression of hepatic genes associated with ureagenesis (carbamoyl phosphate synthetase [CPS]) and steroid catabolism (CYP2C19 and CYP3A4) in beef cows receiving soybean meal supplementation daily (D; $n = 14$), soybean meal supplementation 3 times/week (3WK; $n = 14$), or soybean meal supplementation once/week (1WK; $n = 14$)¹

Item	Treatment			SEM	P-value
	D	3WK	1WK		
Uterine flushing pH ²	6.14	6.13	6.20	0.03	0.10
mRNA expression, relative fold change ³					
CPS	4.22 ^a	4.59 ^a	7.21 ^b	0.69	<0.01
CYP2C19	10.32	10.67	8.24	1.16	0.25
CYP3A4	13.82	5.65	9.82	3.24	0.15

^{a,b}Within rows, values with different superscript differ ($P \leq 0.05$).

¹Cows were assigned to a 3×3 Latin square design, containing 3 periods of 21 d. Within each period, cows were assigned to the following estrus synchronization protocol: 100 μ g of GnRH + controlled internal drug release device (CIDR) containing 1.38 g of progesterone on d 1, 25 mg of PGF_{2 α} on d 8, and CIDR removal + 100 μ g of GnRH on d 11. Soybean meal was individually supplemented at a daily rate of 1 kg/cow (as-fed basis). Moreover, 3WK was supplemented on d 0, 2, 4, 7, 9, 11, 14, 16, and 18, whereas 1WK was supplemented on d 4, 11, and 18.

²Uterine flushing fluid pH was collected 28 h after supplements were offered on d 11 and 18, according to Hersom et al. (2010).

³Liver samples were collected via needle biopsy (Arthington and Corah, 1995) 28 h after supplement feeding on d 11 and at 0, 4, and 28 h relative to supplement feeding on d 18 according to the procedures described by Arthington and Corah (1995). Samples were analyzed for mRNA expression of CPS-I (h 28) and CYP2C19 and CYP3A4 (h 0 and 4 on d 18).

Moreover, mRNA translation into the active enzyme requires time (Clancy and Brown, 2008); hence, one can speculate that CPS-I expression assessed 28 h after supplementation reflected the ureagenesis rate at h 36 after supplementation, when PUN concentrations were similar between 3WK and D and reduced for both treatments compared with 1WK (Fig. 1).

A tendency for a treatment effect was detected ($P = 0.10$) for uterine flushing pH, given that 1WK tended to have greater ($P \leq 0.10$) flushing pH compared with D and 3WK whereas flushing pH was similar ($P = 0.79$) between D and 3WK (Table 2). Previous research reported that uterine pH was negatively associated with protein intake and PUN concentrations in cattle (Elrod et al., 1993; Elrod and Butler, 1993; Hammon et al., 2005). Hence, excessive protein intake has been associated with impaired reproductive performance in females (Butler et al., 1996), given that decreased uterine pH can result in loss of sperm viability and embryonic competence (Pouysségur et al., 1984; FitzHarris and Baltz, 2009). Conversely, Grant et al. (2013) fed diets containing different levels of protein (ranging from 10 to 16% CP) to mature beef cows and reported that PUN concentrations and uterine pH were greater for cows

fed the 16% CP diet compared with cohorts receiving the 10% CP diet. These latter results support differences detected herein on protein intake during the day of supplementation and subsequent PUN concentrations and uterine flushing pH in 1WK compared with 3WK and D. In fact, urea and ammonia have positive charges and alkaline properties when in solution (Haynes, 2014). Therefore, neither of these compounds is expected to reduce pH of biological neutral environments. Nevertheless, the exact mechanism or mechanisms by which excessive protein intake modulates uterine pH in cattle remains unknown and deserves further investigation, particularly because alteration of uterine pH in response to protein intake may be unique to this organ (Elrod and Butler (1993).

Parameters Associated with Meal Size

No treatment effects were detected ($P = 0.97$) on plasma glucose (Table 3). Although plasma glucose concentrations are directly associated with nutrient intake (Vizcarra et al., 1998), the supplement used herein was based on soybean meal, which contains limited amounts of carbohydrates and is not considered a glucogenic precursor to cattle (NRC, 1996). Hence, the greater soybean meal intake of 1WK and 3WK following a supplementation event was likely not sufficient to impact plasma glucose concentrations, at least within 12 h after supplementation, compared with D. Accordingly, previous research from our group (Cooke et al., 2008; Moriel et al., 2012) and other research groups (Drewnoski et al., 2014) also reported similar plasma glucose in beef cattle assigned to different supplementation frequencies during the days in which all cattle were supplemented.

A treatment effect was detected ($P = 0.01$) for plasma insulin, given that plasma insulin concentrations were greater ($P \leq 0.03$) for D and 3WK compared with 1WK but similar ($P = 0.58$) between D and 3WK (Table 3). These results were unexpected because circulating insulin concentrations are also associated with nutrient intake and regulated by blood glucose concentrations (Vizcarra et al., 1998). Nevertheless, cattle consuming excessive protein may shift AA required for synthesis of insulin (Reed et al., 2007), such as aspartate and arginine, to support the urea cycle (Lobley et al., 1995). Therefore, the greater ureagenesis rate of 1WK compared with 3WK and D, based on the rapid increase in PUN concentrations after supplement feeding and past research documenting increased ureagenesis with infrequent supplementation (Wickersham et al., 2008), may have impaired pancreatic insulin synthesis and subsequent circulating concentrations of this hormone. In addi-

Table 3. Mean plasma concentration glucose, insulin, and progesterone (P_4) of beef cows receiving soybean meal supplementation daily (D; $n = 14$), soybean meal supplementation 3 times/week (3WK; $n = 14$), or soybean meal supplementation once/week (1WK; $n = 14$)^{1,2}

Item	Treatment			SEM	P-value
	D	3WK	1WK		
Glucose, mg/dL	60.35	60.38	60.20	1.06	0.97
Insulin, μ U/mL	4.57 ^a	4.76 ^a	3.80 ^b	0.44	0.01
P_4 , ng/mL	2.78	2.53	2.84	0.32	0.65

^{a,b}Within rows, values with different superscript differ ($P \leq 0.10$).

¹Cows were assigned to a 3×3 Latin square design, containing 3 periods of 21 d. Within each period, cows were assigned to the following estrus synchronization protocol: 100 μ g of GnRH + controlled internal drug release device (CIDR) containing 1.38 g of P_4 on d 1, 25 mg of PGF_{2 α} on d 8, and CIDR removal + 100 μ g of GnRH on d 11. Soybean meal was individually supplemented at a daily rate of 1 kg/cow (as-fed basis). Moreover, 3WK was supplemented on d 0, 2, 4, 7, 9, 11, 14, 16, and 18, whereas 1WK was supplemented on d 4, 11, and 18.

²Blood samples were collected from 0 (before) to 72 h after supplementation on d 11 and 18 and analyzed for plasma urea-N. Samples collected from 0 to 12 h were also analyzed for plasma glucose, insulin, and P_4 (d 18 only).

tion, insulin has been shown to inhibit the expression of urea cycle enzymes, including CPS-I (Kitagawa et al., 1985; Morris, 1992). Therefore, the greater mRNA expression of CPS-I in 1WK compared with 3WK and D (Table 2) may also be attributed to treatment effects detected for plasma insulin concentrations (Table 3).

No treatment effects were detected ($P = 0.65$) on plasma P_4 concentrations (Table 3). Similarly, no treatment effects were detected on hepatic mRNA expression of CYP2C19 ($P = 0.25$) and CYP3A4 ($P = 0.15$; Table 2). These results indicate that infrequent protein supplementation did not impact plasma P_4 concentrations, the hormone required for pregnancy establishment and maintenance (Spencer and Bazer, 2002), within 12 h after supplements were offered. One of the hypotheses of this experiment was that beef cows supplemented infrequently would have reduced plasma P_4 concentrations following a supplementation event, which could negatively impact cattle reproductive efficiency given that circulating P_4 concentration 7 d after AI impacts embryonic viability and subsequent pregnancy rates (Mann and Lamming, 2001; Demetrio et al., 2007). This hypothesis was developed based on the premise that large meal size reduces circulating P_4 concentrations by increasing hepatic blood flow and subsequent hepatic P_4 catabolism by CYP2C19 and CYP3A4 (Sangsritavong et al., 2002). Supporting our rationale, Vasconcelos et al. (2003) demonstrated that dairy cows receiving 100% of their diet in a single meal had reduced P_4 concentrations compared with cohorts fed their diets in multiple but smaller meals. Similarly, Cooke et al. (2007) reported that beef females

receiving energy-based supplements 3 times weekly had reduced circulating P_4 concentrations after supplementation. In the present experiment, the lack of differences in plasma P_4 concentrations among treatments suggest that the increased meal size resultant from reduced supplementation frequency, at the rate used herein, was not sufficient to impact hepatic steroid catabolism and subsequent plasma P_4 concentrations. Perhaps a greater increase in meal size (Vasconcelos et al., 2003) or inclusion of highly fermentable substrates such as energy ingredients (Cooke et al., 2007) are required to impact these parameters. Last but not least, insulin impacts plasma P_4 concentrations by stimulating luteal P_4 synthesis (Spicer and Echtenkamp, 1995) and reducing hepatic P_4 catabolism by CYP2C19 and CYP3A4 (Cooke et al., 2012; Vieira et al., 2013). Therefore, the difference in plasma insulin concentrations of D and 3WK compared with 1WK following a supplementation event was not sufficient to elicit a similar effect on plasma P_4 concentrations. Accordingly, research from Lemley et al. (2008) suggested that there might be a threshold in circulating insulin concentrations that must be reached to alleviate expression of CYP2C19 and CYP3A4 and consequently increase circulating P_4 concentrations.

Overall Conclusion

Supplementing protein to beef cows as infrequent as once weekly increased PUN concentrations and hepatic mRNA expression of the rate-limiting enzyme of the urea cycle but did not reduce uterine flushing pH or circulating P_4 concentrations after supplements were offered. Nevertheless, additional research within this subject is warranted, including experiments designed to determine whether the frequency of protein supplementation to lactating beef cows during the breeding season can be reduced, such as 3 times or once weekly, without impairing pregnancy rates.

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