Effects of intraruminal urea-nitrogen infusions on feed intake, nitrogen utilization, and milk yield in dairy cows

S. Ahvenjärvi^{*1} and P. Huhtanen[†]

^{*}Natural Resources Institute Finland (Luke), Milk Production, FI-31600 Jokioinen, Finland

[†]Department of Agricultural Research for Northern Sweden, Swedish University of Agricultural Sciences (SLU), S-90183 Umeå, Sweden

ABSTRACT

The objective of this study was to determine the effects of supplementation of protein deficient diet with increasing amounts of urea-N on feed intake, milk yield, rumen fermentation, and nutrient digestibility in dairy cows. The hypothesis was that low rumen ammonia-N concentrations provide suboptimal conditions for rumen microbes and these conditions can be alleviated by urea-N that increases rumen ammonia-N concentrations. To evaluate this hypothesis, the diet was formulated slightly deficient with respect to rumen-degradable protein. To supplement the diet with rumen degradable N, 5 levels of urea-N (0, 17, 33, 49, and 66 g/d) were continuously infused into the rumen of 5 dairy cows according to a 5 × 5 Latin square. Increasing levels of urea-N infusion increased N intake and N excretion in urine and feces in a linear manner and tended to increase milk and milk protein yields. Feed intake and fiber digestibility were not affected by urea-N infusion levels. Rumen ammonia-N concentrations remained low (3.5 mg/100 mL) and did not respond to urea-N infusions levels between 0 to 49 g/d, whereas the highest level of urea-N (66 g/d) increased rumen ammonia-N concentration to 5.1 mg/100 mL (quadratic effect). These observations suggested that rumen microbes efficiently captured ammonia-N from rumen fluid until sufficient intracellular ammonia-N concentrations were attained, after which ammonia-N concentrations started to increase in extracellular rumen fluid. In contrast, milk urea-N concentrations increased in a curvilinear manner (cubic effect) from 4.4 to around 6 mg/100 mL for the medium levels of urea-N and then to 7.9 mg/100 mL for the highest level of urea-N infusion. The current results indicated that 18% of supplementary N intake was secreted in milk

and 53% in urine. In spite of low rumen ammonia-N concentrations observed for the basal diet, it was estimated that only 43% of supplementary N was captured by rumen microbes. Estimated true digestibility for supplementary N (93%) provided further evidence that urea-N stimulated microbial N synthesis. The current results indicate that rumen ammonia-N concentration was an insensitive indicator of N deficiency at low levels of diet CP, whereas milk urea-N was responsive to diet CP concentrations at all urea-N infusion levels.

Key words: urea, dairy cow, nitrogen, rumen

INTRODUCTION

The main principle in formulation of diets for dairy cows has been to provide a ration that allows individuals to satisfy their nutritional requirements. In an attempt to account for the N requirements of the rumen microbes and protein requirements of the cow, a distinction between RDP and RUP has been made in the current feed evaluation systems. However, the success of assigning dietary protein to these 2 fractions has been meager (Schwab et al., 2005). Arguably, dairy cows do not have absolute requirements for the amount of MP because they can adapt to different levels of nutrition by adjusting their feed intake and milk yield as required to maintain a balance between nutrient supply and body requirements (Vérité and Delaby, 2000; Huhtanen et al., 2011b). The optimum amount of CP in the diet depends on definition because the optimum for milk yield is often very different from the optimum for N use efficiency (Huhtanen et al., 2011a). Supplementation of dairy cow diets with protein feeds has often resulted in positive production responses in terms of milk and milk protein yield (Vérité and Delaby, 2000; Ipharraguerre and Clark, 2005; Huhtanen et al., 2011a). In spite of positive milk yield responses to increases in CP intake, the N use efficiency (milk N/N intake) has consistently decreased (Castillo et al., 2001; Nursoy et al., 2018). The main reason for such decreases is that a large proportion of feed protein is degraded in the rumen and only a small proportion enters the omasal

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¹Corresponding author: seppo.ahvenjarvi@luke.fi

canal as undegradable feed protein (Broderick et al., 2010). Furthermore, increasing the MP to energy ratio above recommendations resulted in linear increases in urine N excretion but only minor increases in milk yield (Vérité and Delaby, 2000). Attempts to increase the proportion of RUP in protein feeds have often been successful in enhancing ruminal outflow of nonammonia N, but the effects on milk CP yield have been minor and sometimes even negative (Ipharraguerre and Clark, 2005). Feeding a diet that provides RDP in excess of microbial needs will inevitably decrease the N use efficiency (Broderick, 2003; Huhtanen and Hristov, 2009) but even maintaining the N use efficiency using supplements enriched with RUP seems unrealistic. This has been convincingly demonstrated by studies in which supplementary protein was supplied directly into the abomasum of dairy cows (Choung and Chamberlain, 1993; Guinard et al., 1994). In both studies lowest levels of casein or soya protein isolate infused into the abomasum were efficiently used for milk protein synthesis and either improved or maintained the N use efficiency of the basal diet. However, higher amounts of either casein or soya protein resulted in diminishing utilization for milk protein synthesis (Choung and Chamberlain, 1993; Guinard et al., 1994). In practice such utilization rates of supplementary protein, as achieved using abomasal casein infusions, cannot be attained because of inevitable degradation losses in the rumen, lower intestinal CP digestibility, and less ideal AA composition of RUP supplements. Arguably, the most efficient means of improving the N use efficiency in milk production is to formulate a diet that provides sufficient RDP for rumen microbes but avoids excess supply that is metabolized to ammonia-N, absorbed from the digestive tract, and lost in urine (Huhtanen and Hristov, 2009). A previous study in lactating dairy cows given pulse doses of ^{15}N labeled ammonia-N and grass silage soluble N indicated that 53 and 62%, respectively, of rumen bacterial N was derived from rumen ammonia-N pool (Ahvenjärvi et al., 2018). These observations suggest that rumen ammonia-N pool plays an important role in rumen N dynamics and NPN sources could be used to complement deficiencies in RDP supply.

The objective of the current study was to assess the critical diet CP and rumen ammonia-N concentrations, below which the microbial N synthesis and fiber digestibility are decreased relative to the optimal conditions. The hypothesis underlying the current study was that feed intake, nutrient digestibility, and milk yield of lactating dairy cows are compromised when offered a diet formulated slightly deficient in terms of RDP. The hypothesis further assumed that suboptimal rumen conditions could be improved by urea-N infusions into

the rumen that increase rumen ammonia-N concentrations.

MATERIALS AND METHODS

Animals, Diet, and Experimental Design

The current experiment was conducted in compliance with the Finnish Act on the Use of Animals for Experimental Purposes and experimental procedures were approved by the National Animal Experiment Board. The effects of intraruminal urea-N infusions were investigated using 5 lactating dairy cows that received a basal diet formulated marginally deficient of RDP. Grass silage was provided as forage because it is the most widely cultivated forage crop in Northern Europe. In addition, our previous study (Ahvenjärvi et al., 2018) indicated that a pulse dose of ammonia-N introduced into the rumen of dairy cows fed grass silage-based diet was efficiently used for microbial N synthesis. Experimental animals were multiparous Finnish Ayrshire dairy cows equipped with 10 cm i.d. rumen cannulas (Bar Diamond Inc., Parma, ID). The cows were on average 77 DIM (SD 12.3) at the beginning of the experiment and weighed 620 kg (SD 35.6). The basal diet was offered 4 times daily at 0600, 0900, 1800, and 2000 h according to appetite allowing at least 5% for the daily refusals. Silage was prepared from a primary growth of a mixture of timothy (*Phleum pratense*) and meadow fescue (*Festuca pratensis*) grass species. Grass was cut and conditioned using a mower-conditioner and allowed to wilt for a few hours before it was harvested using a precision chopper. To restrict the extent of silage fermentation, silage additive [AIV 2 Plus (Formic acid 77%, Ammonium formate 5.5%) Taminco Finland Oy, Oulu, Finland] was applied onto grass at harvest at a rate of 5 L/t. Grass was ensiled in bunker silos until mixed with concentrates to prepare TMR that contained (per kg of DM) 511 g of grass silage, 156 g of wheat, 152 g of oats, 147 g of molassed sugar beet pulp, 20 g of solvent-extracted rapeseed meal, and 15 g of mineral and vitamin premix (Rehumelica Oy, Vaasa, Finland).

The experimental treatments consisted of 5 levels of urea-N (0, 17, 33, 49, and 66 g/d of N; Sigma-Aldrich Chemie GmbH, Steinheim, Germany) dissolved in 6 L of water and infused into the rumen through a rumen cannula using a peristaltic pump (Watson-Marlow 502 S, Falmouth, UK). Urea-N was administered by means of water infusion to avoid palatability problems often encountered with urea-N-containing diets and to ensure constant daily doses. Treatments were randomly allocated to each cow according to a 5×5 Latin square

change-over design. The experiment consisted of 5 periods each that lasted for 14 d. Feed intake recorded from d 10 to 14 was used to compare treatment effects.

Sampling Procedures

Nutrient digestibility and N excretion in urine were determined based on total collection of feces and urine conducted over 3 d from d 11 to 13 of each period. Rumen ammonia-N and VFA concentrations were determined on d 14 based on 8 samples collected at 1.5-h intervals from 0600 to 1630 h. Rumen fluid samples of approximately 200 mL were aspirated via the rumen cannula using a perforated plastic tube. Samples were immediately filtered through one layer of cheesecloth and measured for pH. For VFA analysis, 5.0 mL of rumen fluid was treated with 0.5 mL of saturated HgCl_2 and 2.0 mL of 1 M NaOH solution. For ammonia-N analysis, 0.3 mL of 50% H_2SO_4 was added to 15 mL of rumen fluid. Samples were stored at -20°C until analyzed. Milk yields measured from d 10 to 14 daily were used to compare treatment effects. Milk composition and MUN concentrations were analyzed from samples collected from 4 consecutive milkings from d 11 to 13.

Chemical Analysis

Feed and fecal samples were dried in a forced-air oven at 60°C and milled for chemical analysis using a cutter mill with a 1-mm screen. Residual DM concentration of dried and ground samples was determined in a forced-air oven at 105°C for 16 h. Silage DM concentration was corrected for the loss of volatile compounds as described by Huida et al. (1986). Ash concentration was determined in a muffle furnace according to AOAC method 942.05 (AOAC International, 2012). Nitrogen concentration in fresh samples of feces and urine was determined by the Kjeldahl method using CuSO_4 as a catalyst. Nitrogen concentration in dry feed samples was determined using a Dumas-type elemental N analyzer (Leco FP-428, Leco Corporation, St. Joseph, MI). Starch concentration in feed ingredients was determined as described by Salo and Salmi (1968). The NDF concentration was analyzed in the presence of Na_2SO_3 using crucibles as described by Van Soest et al. (1991). Heat-stable α -amylase was used for starch-containing samples. Indigestible NDF in feeds and feces was determined based on 12-d in situ incubations in the rumen of dairy cows as described by Ahvenjärvi et al. (2006). Grass silage OM digestibility was determined based on pepsin-cellulase OM solubility as described by Nousiainen et al. (2003) and modified by Huhtanen et al. (2006). Concentration of digestible OM (D-value, g/

kg of DM) in grass silage was estimated using the equation proposed by Huhtanen et al. (2006): $\text{D-value (g/kg of DM)} = [1000 - \text{ash (g/kg of DM)}] \times [0.077 + 0.86 \times \text{OM solubility (g/g of OM)}]$. Milk urea-N concentration was calculated from a difference in ammonia-N concentration between unhydrolyzed sample and that hydrolyzed with urease. Ammonia-N concentrations in rumen fluid were determined by direct colorimetric method (McCullough, 1967). Volatile fatty acid concentrations in grass silage and rumen fluid were determined by GC as described by Huhtanen et al. (1998). Concentrations of lactic acid and water-soluble carbohydrates in grass silage were determined as described by Haacker et al. (1983) and Somogyi (1945), respectively. Ethanol concentration was measured with an enzymatic kit (catalog no. 981680, KONE Instruments Corporation, Espoo, Finland) using the selective clinical chemistry analyzer Pro 981489 (KONE Instruments Corporation). Milk samples were analyzed for CP, fat, and lactose concentration using an infrared milk analyzer (MilkoScan 133B, Foss Electric, Hillerød, Denmark). Milk true protein concentration was calculated as $\text{milk CP\%} - (\text{MUN\%} \times 6.38)$.

Statistical Analysis

Experimental data were analyzed using PROC MIXED of SAS 9.2 software (SAS Institute Inc., Cary, NC). The effect of urea-N treatment on feed intake, digestibility, milk yield and composition, and rumen fermentation was estimated using the following model:

$$Y_{ijk} = \mu + A_i + P_j + U_k + \varepsilon_{ijk},$$

where μ is the overall mean; A_i is the random effect of animal; P_j and U_k are the fixed effects of period and urea-N treatment, respectively; and ε_{ijk} is the residual variation. Sums of squares were further separated using orthogonal contrasts into single degree of freedom comparisons to test the significance of linear, quadratic, and cubic effects of urea-N infusion levels.

The effects of urea-N treatment on rumen ammonia-N concentrations were evaluated using a model for repeated measurements with first-order autoregressive covariance structure:

$$Y_{ijkl} = \mu + A_i + P_j + U_k + A_i \times P_j \times U_k + T_l + A_i \times T_l + P_j \times T_l + U_k \times T_l + e_{ijkl},$$

where μ , A_i , P_j , and U_k are as previously described; T_l is the sampling time; $A_i \times P_j \times U_k$, $A_i \times T_l$, $P_j \times T_l$, and $U_k \times T_l$ are the interactions; and e_{ijkl} is the residual variation.

Table 1. Chemical composition of grass silage and concentrate ingredients

Item	Grass silage ¹	Wheat	Oats	Sugar beet pulp	Rapeseed meal	Mineral and vitamin premix ²	TMR
DM, g/kg	259	855	853	892	912	996	395
Composition, g/kg of DM							
OM	931	979	961	924	925	—	906
N	22.6	16.5	18.8	18.1	60.2	0.5	20.3
NDF	523	112	265	314	260	NA ³	364
iNDF ⁴	66	12	115	20	112	NA	58
Starch	NA	683	465	52	20	NA	185

¹Fermentation quality: pH 4.0; in DM, g/kg: lactic acid 63, acetic acid 20, propionic acid 0.2, butyric acid 0.5, ethanol 6.9, water-soluble carbohydrates 74; in total N, g/kg: soluble N 621, ammonium-N 55.

²Mineral and vitamin composition: Ca, 205 g/kg; P, 27 g/kg; Na, 85 g/kg; Mg, 75 g/kg; vitamin A, 120,000 IU/kg; vitamin D₃, 25,000 IU/kg; vitamin E, 340 mg/kg; Mn, 350 mg/kg; Zn, 1,460 mg/kg; Cu, 270 mg/kg; I, 39 mg/kg; Co, 27 mg/kg; and Se, 20 mg/kg.

³NA = not analyzed.

⁴Indigestible neutral detergent fiber.

RESULTS

Chemical Composition of Feeds

Chemical composition of feed ingredients and TMR are presented in Table 1. Grass silage for the current study was prepared with an objective of high digestibility (707 g/kg of DM of digestible OM) with moderate CP concentration (141 g/kg of DM). The fermentation characteristics of grass silage were typical for slightly wilted grass (DM concentration 259 g/kg) ensiled in a bunker silo using formic acid preservatives. The concentration of water-soluble carbohydrates was high in grass silage, whereas that of fermentation acids was low (84 g/kg of DM) with lactic acid being the predominant component (74% of total acids). Wheat, oats, and sugar beet pulp were selected as concentrate ingredients to formulate a highly digestible ration with moderate CP concentration. Rapeseed meal was included in TMR to adjust the CP to the targeted concentration of 130 g/kg of DM. Owing to slightly lower CP concentration of grass silage offered to the cows than determined before the experiment the CP concentration of the basal diet was 127 g/kg of DM. According to the Finnish feed evaluation system (Finnish Feed Tables and Nutrient Requirements, 2018), the basal diet provided slightly less RDP than required by the rumen microbes with estimated rumen CP balance of -58 g/d (SD 13) or -2.6 g/kg of DM (SD 0.5). Supplementary N provided as urea-N increased the diet CP concentration from 127 to 131, 136, 140, and 145 g/kg of DM for the urea-N infusion levels of 17, 33, 49, and 66 g/d, respectively. As predicted based on the Finnish feed evaluation system, the amount of MP provided by the basal diet was equal to the estimated MP requirements (MP intake – MP requirement = -1 g/d; SEM 42) when a reduction in rumen microbial CP synthesis was assumed to be equal to the negative rumen CP balance. However, urea-N in-

fusions did not have an effect (linear effect $P = 0.38$) on estimated MP balance (MP intake – MP requirement) because MP requirements tended to increase ($P = 0.08$) as a result of a tendency for increases in milk yield.

Feed Intake and Milk Yield

The level of urea-N infusion had no effect on DM, OM, digestible OM, or NDF intake or digestibility of these chemical components (Table 2). Nitrogen intake from the basal diet was not affected by the treatment, but urea-N infusions increased total N intake ($P < 0.01$), and N excreted in urine ($P < 0.01$) and feces ($P = 0.05$) in a linear manner. Increases in fecal N output were associated with increases in fecal N concentrations ($P = 0.02$). Urea-N infusions tended to decrease the N use efficiency (linear effect, $P = 0.07$). The N balance of cows was slightly positive for the lower levels of urea-N infusion (0 to 49 g of urea-N) but increased for the highest urea-N level as indicated by the significant cubic effect for the urea-N infusions ($P = 0.04$).

Milk yield ($P = 0.08$), milk CP yield ($P = 0.08$), and true protein yield ($P = 0.10$) tended to respond in a linear manner to increasing levels of urea-N infusions (Table 3). Milk urea-N concentrations increased in a curvilinear manner (cubic effect, $P = 0.05$) from 4.4 mg/100 mL for the lowest level of urea-N infusion to around 6 mg/100 mL for the medium levels of infusion (17 to 49 g/d), followed by a substantial increase up to 7.9 mg/100 mL for the highest level of infusion (66 g/d). No significant effects on ECM, fat or lactose yield, or milk composition were observed.

Rumen Fermentation

Ruminal ammonia-N concentrations exhibited postprandial peaks at 1.5 h after the 0600 h meal that

Table 2. Effects of urea infusion on feed intake and nutrient digestibility

Item	Urea infusion level, g/d					SEM	Orthogonal contrast		
	0	17	33	49	66		Linear	Quadratic	Cubic
Nutrient intake, kg/d									
DM	22.5	22.7	22.5	22.6	23.4	0.84	0.35	0.46	0.56
OM	20.4	20.6	20.4	20.5	21.2	0.76	0.35	0.46	0.57
Digestible OM	14.2	14.5	14.3	14.4	14.9	0.56	0.31	0.62	0.47
NDF	8.20	8.26	8.18	8.24	8.52	0.305	0.35	0.44	0.59
N, g/d									
Basal diet	458	461	457	460	475	17.0	0.35	0.46	0.57
Total	458	478	490	508	541	17.1	<0.01	0.46	0.55
Nutrient digestibility, kg/kg									
OM	0.694	0.704	0.701	0.700	0.703	0.0056	0.35	0.56	0.31
NDF	0.549	0.559	0.562	0.559	0.564	0.0111	0.31	0.61	0.58
N	0.614	0.625	0.612	0.603	0.612	0.0075	0.32	0.99	0.09
N excretion									
In urine, g/d	109	119	131	145	151	5.1	<0.01	0.81	0.56
In feces, g/d	177	173	177	182	184	5.7	0.05	0.36	0.29
Fecal N, g/kg of DM	25.2	25.3	25.8	26.3	26.0	0.53	0.02	0.50	0.24
N use efficiency, g/g of intake	0.364	0.359	0.346	0.358	0.333	0.0106	0.07	0.69	0.41
N balance, g/d	5	14	12	0	26	7.1	0.22	0.37	0.04

reached 9.2 to 9.8 mg/100 mL for all urea-N infusion levels (Figure 1). After the peak levels ruminal ammonia-N concentrations declined steadily, reaching the lowest levels (0.4 mg/100 mL) at 9 h after the morning meal for the control treatment (0 g/d). Supplementary urea-N infusions did not increase ruminal ammonia-N concentrations relative to the control treatment except for the highest level of infusion (66 g/d). Lowest ruminal ammonia-N concentrations remained between 0.4 to 0.9 mg/100 mL for the urea-N infusion levels between 0 to 49 g/d but for the highest level of urea-N infusion (66 g/d) the lowest ammonia-N concentrations did not decrease below 2.2 mg/100 mL (Figure 1).

Irrespective of the urea-N infusion level, mean rumen ammonia-N concentrations remained very low for lactating dairy cows (between 3.1 to 5.1 mg/100 mL; Table 4). Nonetheless, urea-N had a curvilinear effect (quadratic effect, $P = 0.01$) on rumen ammonia-N concentrations as no effects were observed for the lower levels (0 to 49 g/d), whereas for the highest level of urea-N infusion (66 g/d) the ammonia-N concentrations sharply increased (Table 4). The curvilinear effect of supplementary urea-N is evident in the relationship between diet CP concentration and rumen ammonia-N concentrations (Figure 2). Urea-N infusion levels had no effect on rumen pH, concentration of VFA in the

Table 3. Effects of urea infusion on milk production and composition

Item	Urea infusion level, g/d					SEM	Orthogonal contrast		
	0	17	33	49	66		Linear	Quadratic	Cubic
Milk, kg/d	32.2	32.9	33.0	34.8	33.7	1.59	0.08	0.61	0.41
ECM, ¹ kg/d	32.5	34.2	31.8	34.1	33.8	1.39	0.46	0.88	0.65
Milk composition, %									
Fat	4.15	4.35	3.79	3.91	4.04	0.184	0.21	0.39	0.14
CP	3.31	3.34	3.29	3.34	3.42	0.094	0.27	0.44	0.63
True protein ²	3.28	3.30	3.25	3.30	3.37	0.095	0.39	0.46	0.69
Lactose	4.63	4.70	4.64	4.62	4.63	0.059	0.45	0.41	0.10
MUN, mg/100 mL	4.43	5.90	5.87	6.40	7.89	0.433	<0.01	0.67	0.05
Yield of milk components, kg/d									
Fat	1.33	1.43	1.25	1.35	1.35	0.062	0.88	0.66	0.36
CP	1.06	1.10	1.09	1.16	1.15	0.056	0.08	0.98	0.75
True protein	1.05	1.09	1.07	1.15	1.13	0.056	0.10	0.98	0.72
Lactose	1.49	1.55	1.53	1.61	1.57	0.080	0.15	0.55	0.74

¹ECM = milk yield $\times$ (383 $\times$ fat % + 242 $\times$ protein % + 165.4 $\times$ lactose % + 20.7)/3,140 (Sjaunja et al., 1990).

²True protein = CP% - (milk urea nitrogen% $\times$ 6.38).

Figure 1. The effect of urea infusion level on diurnal pattern of ruminal ammonia-N concentrations (mg/100 mL).

rumen or the molar proportions of individual VFA with the exception of butyrate that decreased and the propionate:butyrate ratio that increased as the urea-N infusions levels increased (linear effects, $P = 0.05$).

Urea-N Utilization

Nitrogen excretion in urine and feces exhibited consistent linear responses to urea-N infusion levels. When N intake was plotted against the amount of N excreted

in urine, the slope indicated that proportionally 53% ($R^2 = 0.94$, Figure 3) of N intake was excreted in urine. The relationship between N intake and true protein N output in milk suggests that proportionally 18% ($R^2 = 0.74$, Figure 4) of urea-N was used for milk protein synthesis. When the diet CP concentration is plotted against digestible CP concentration (Figure 5), the slope provides an estimate for true digestibility of supplementary N and the intercept represents an estimate for the amount of metabolic fecal CP excreted per unit

Table 4. Effects of urea infusion on rumen fermentation characteristics

Item	Urea infusion level, g/d					SEM	Orthogonal contrast		
	0	17	33	49	66		Linear	Quadratic	Cubic
pH	6.5	6.4	6.6	6.5	6.5	0.12	0.84	0.94	0.40
NH ₃ -N, mg/100 mL	3.8	3.6	3.1	3.3	5.1	0.41	0.09	0.01	0.16
Total VFA, mmol/L	115	118	113	118	119	2.6	0.38	0.45	0.58
Molar proportion of VFA, mmol/mol									
Acetate	647	629	642	642	637	7.2	0.78	0.58	0.10
Propionate	181	195	187	194	197	6.1	0.13	0.69	0.35
Isobutyrate	6.4	5.9	6.2	6.3	5.8	0.32	0.34	0.74	0.13
Butyrate	135	140	133	128	130	5.6	0.05	0.73	0.11
Isovalerate	8.6	8.4	8.7	8.3	8.1	0.83	0.46	0.71	0.87
Valerate	13.7	14.1	13.6	13.6	13.7	0.53	0.63	0.92	0.50
Caproate	8.6	8.0	9.1	8.3	9.0	0.73	0.65	0.74	0.96
Pr/Bu ¹	1.35	1.42	1.44	1.53	1.52	0.081	0.05	0.72	0.77

¹Propionate/butyrate.

Figure 2. The relationship between diet CP (g/kg of DM) and rumen ammonia-N concentrations (mg/100 mL).

of feed intake (Lucas test). The slope of the regression indicated true digestibility of 0.93 for supplementary CP intake and 39 g of metabolic fecal CP per kilogram of DMI.

DISCUSSION

Nitrogen Utilization

Dairy businesses are confronted with increasing pressures not only to be economically competitive but also to produce milk that is accepted as ecologically sustainable. To respond to these challenges, dairy nutritionists need reliable and readily available tools to control the daily ration in terms of adequate nutrient supply rela-

Figure 3. The effect of N intake (g/d) on the amount of N excreted in urine (g/d).

Figure 4. The effect of N intake (g/d) on the amount of true protein N output in milk (g/d).

tive to cows' requirements. Ration CP and milk urea-N concentrations are simple diagnostic tools (Broderick and Clayton, 1997), but they have been somewhat overlooked because the current models have emphasized the role of RUP as a source of supplementary MP, whereas RDP has been considered a homogeneous source of N for microbial protein synthesis without any distinction in effectiveness between NPN and true protein. However, previous studies have demonstrated that RDP from true protein sustains higher efficiency

Figure 5. The plot of digestible CP concentration against dietary CP concentration to assess true CP digestibility of incremental N intake.

of microbial protein synthesis than NPN (Broderick and Reynal, 2009). Critical evaluations of the current protein systems have suggested that the performance of complex approaches to predict MP intake may not be superior to simple systems based on ME and diet CP intake (Schwab et al., 2005).

In the current study the relationship between N intake and true protein N output in milk suggested N use efficiency of 18% for supplementary N. Although microbial N synthesis in the rumen was not currently measured, increases in milk true protein yield and fecal N output indicated that supplementary N was used by the rumen microbes. Assuming 80% of rumen microbial N entering the intestines is true protein, digestibility of this true protein is 80% (NRC, 2001), and the efficiency of MP use of for milk protein synthesis is 67% (NRC, 2001), the efficiency of microbial protein utilization for milk protein synthesis is 43%. Assuming that in the current study 18% of supplementary urea-N was transferred to milk true protein N, the proportion used by the rumen microbes ($0.18/0.43$) suggests that 42% of urea-N was captured by the rumen microbes. This figure represents both immediate capture of urea-N to microbial N and recycled urea-N that re-entered the forestomach via the hepatic ornithine cycle. The current estimate of ruminal utilization of supplementary urea-N is in agreement with the mean values for urea-N kinetics estimated by Lapierre and Lobley (2001). These authors estimated that typically 33% of hepatic urea-N flux is eliminated in urine, whereas 67% enters the digestive tract. Of this latter, 50% is reabsorbed as anabolic-N (microbial N), whereas 40% is reabsorbed as ammonia-N and 10% is lost in feces. However, the efficiency of urea recycling into the forestomach is dependent on the diet CP concentration as previously demonstrated by Kristensen et al. (2010). These authors observed a substantial increase in arterial urea extraction across the rumen wall when cows were switched from high N to low N diet (17.1 vs. 12.9% CP).

In the current study, one diet was offered to the animals and supplementary urea-N was provided directly into the rumen to avoid confounding effects due to changes in diet composition. Therefore, the Lucas test was expected to indicate 100% true digestibility for supplementary urea-N in case it was completely absorbed from the digestive tract without being used for microbial CP synthesis. Alternatively, in a case that the Lucas test suggested lower than 100% digestibility for urea-N, it was expected to indicate microbial utilization of supplementary N because microbial cells are incompletely digested and increase fecal N excretion per unit of DMI. A linear increase in fecal CP concentrations was currently observed. The Lucas test indicated true

digestibility of 93% for supplementary N and 39 g/kg of DM for fecal metabolic CP. These figures are in line with true digestibilities between 92 to 96% and fecal metabolic CP between 26 to 39 g/kg of DM estimated for supplementary protein provided as untreated canola meal, soybean meal, and the mixture of soybean and fishmeal (Huhtanen et al., 2011a). Similar total-tract digestibility estimates for urea-N and protein feeds provide evidence that supplementary urea-N was partly used for microbial N synthesis. If increases in diet CP concentrations are attained by incremental urea-N infusion levels, the digestibility of supplementary N should be complete unless microbial N synthesis either in the rumen or in the hindgut (i.e., cecum and colon) is increased. Assuming 82% true digestibility for rumen microbial CP (Storm et al., 1983) and 100% digestibility for the fraction of supplementary N that was not used for microbial N synthesis, an estimate of 39% for the proportion of urea-N captured by the rumen microbes is indicated. This estimate agrees with the value (42%) estimated above based on the use of supplementary N for milk N synthesis. Albeit in good agreement, both estimates are based on several assumptions such that their accuracy should be regarded with caution.

The efficiency of urea-N use for milk true protein synthesis in the current study was higher (18%) than the marginal milk protein yield responses (g/kg increase in CP intake) estimated for rapeseed meal CP (13.5%) or soybean meal CP (10%) in a meta-analysis by Huhtanen et al. (2011a). An obvious reason for the high marginal responses to supplementary urea-N in the current study was the low dietary CP concentration because marginal responses in milk protein yield tend to decline as a linear function of diet CP concentration ($55.63 \text{ g/d} - 2.52 \text{ g/d} \times \text{CP\%}$; NRC, 2001). Replacement of corn with soybean meal in dairy cow ration increased diet CP concentration from 135 to 165 g/kg of DM and improved milk protein yield with a marginal efficiency of 98 g/kg increase in CP intake (Olmos Colmenero and Broderick, 2006). However, further increases in the amount of soybean meal failed to improve milk protein yield. The diminishing returns often observed in protein supplementation studies can be attributed, at least partly, to poor utilization of excess ammonia-N released from ruminal protein degradation at high diet CP concentrations. Excess ammonia-N is absorbed from the digestive tract into plasma, converted to urea in the liver, recycled back into the gut or filtered from plasma by the kidneys, and excreted in urine (Lobley et al., 2000). The fractional transfer rate of urea into the gut is dependent on the diet CP concentration such that at low CP concentrations higher proportions of plasma urea are recycled into the gut and used for mi-

crobial synthesis, whereas at high CP concentrations higher proportions of plasma urea are eliminated from the body in urine (Wickersham et al., 2008).

Previous findings have suggested that plasma urea transport across the gut epithelia is directly proportional to plasma urea concentrations but also affected by the nutritional status of the animal such that epithelial gut permeability decreases with increasing N status of the animal (Kristensen et al., 2010). Wickersham et al. (2008) studied the effects of incremental casein in steers fed low-quality prairie hay (CP 47 g/kg of DM). The proportion of plasma urea entering the gut decreased linearly from 98 to 83% and the proportion of gut urea-N used by the microbes decreased from 51 to 42% as the diet CP concentration increased from 55 to 91 g/kg of DM by supplementary casein that provided 7.0 to 65.3 g/d of N. In dairy heifers fed isocaloric diets, increasing the CP from 91 to 213 g/kg of DM increased the proportion of the urea-N synthesized in the body that is excreted as urinary urea-N from 15 to 71% and decreased the proportion of N recycled into the gut that was used for microbial protein synthesis from 43 to 6% (Marini and Van Amburgh, 2003). These observations clearly indicate a strong influence of the diet CP concentration on the efficiency of urea recycling and help to explain the high efficiency of supplementary urea-N use observed in the current study.

Rumen Ammonia-N Concentration

In the current study rumen ammonia-N concentrations were determined based on 12-h sampling period covering the hours between 0600 and 1800 h. Hence, it is assumed that these measurements represent the entire 24-h period. To minimize differences in feed intake pattern between day and night time periods, cows were fed at 0600, 0900, 1800, and 2000 h. In the current study rumen ammonia-N concentrations did not exhibit responses to urea-N infusion until the highest level of urea-N was reached. At the lower levels of urea-N infusion (0 to 49 g/d) the average rumen ammonia-N concentrations remained between 3.1 and 3.8 mg/100 mL. This pattern of rumen ammonia-N responses tends to suggest that rumen microbes efficiently captured supplementary ammonia-N until sufficient intracellular ammonia-N concentration was reached. Consistent with this interpretation, Russell and Strobel (1987) observed higher intracellular than extracellular ammonia-N concentrations in mixed rumen bacteria indicative of active ammonia-N transport mechanisms possessed by rumen bacteria. The ammonia-N gradient between extracellular and intracellular space was highest at the low rumen ammonia-N concentrations because bacteria were able to maintain a minimum of 13 mg/100 mL

intracellular ammonia-N concentrations (Russell and Strobel, 1987). The minimum extracellular ammonia-N concentrations (1.0 mg/100 mL) observed by Russell and Strobel (1987) suggested that despite an active transport system rumen bacteria could not completely deplete ammonia-N from the extracellular environment. In contrast to these findings observed *in vitro*, the lowest ammonia-N concentrations in the current study recorded for each individual animal were not more than 0.1 mg/100 mL. Consistent with our results, Marini and Van Amburgh (2003) observed hardly detectable rumen ammonia-N concentrations (average 0.1 mg/100 mL) in Holstein heifers fed a diet with 91 g/kg DM of CP. Hence, it seems evident that mixed rumen bacteria are capable of efficiently depleting ammonia-N from rumen fluid when the rate of ammonia-N release from protein degradation and microbial turnover is lower than microbial uptake. Owing to the active ammonia-N transport systems present in the rumen microbial community, low extracellular ammonia-N concentrations may be an inadequate indicator of microbial intracellular concentrations.

In the current study, an inflection point in the rumen ammonia-N concentrations was achieved at a diet CP concentration of 140 g/kg DM (49 g/d of urea-N). Above this CP concentration extracellular ammonia-N concentrations sharply increased. In a study conducted using steers (Slyter et al., 1979) the response of ruminal ammonia-N concentration to increasing amounts of urea-N infusions into the rumen exhibited a smaller slope between 80 to 169 g/kg DM of CP followed by a steeper slope for ammonia-N concentrations between 169 to 195 g/kg DM of CP. In the study of Marini and Van Amburgh (2003) increases in ruminal ammonia-N concentrations were 3.3 times greater between 118 to 186 g of CP/kg of DM than between 91 to 118 g of CP/kg of DM. These observations could be explained by efficient uptake of rumen ammonia-N into microbial cells until the requirements for optimal growth were achieved. Beyond this point, the rate of ammonia-N release from ruminal protein degradation exceeded the rate of anabolic requirements and ammonia-N started to accumulate in rumen fluid. Considering the curvilinear response in ruminal ammonia-N concentration currently observed, it was somewhat unexpected that the proportion of supplementary urea-N excreted in urine was constant throughout. This could be related to alleged increases in intracellular ammonia-N pool (Russell and Strobel, 1987) that escaped the rumen in microbial N entering the intestines and was absorbed from the intestines, and subsequently excreted in urine.

In the current study, supplementary urea-N had no significant effects on NDF digestibility in spite of very low extracellular ammonia-N concentrations. Slyter

et al. (1979) concluded that near maximum DM and fiber digestibility were attained when the diet CP concentration was 95 g/kg of DM and the average rumen ammonia-N concentration was 1.1 mg/100 mL. Marini and Van Amburgh (2003) observed a reduction in fiber digestibility at a diet CP concentration of 91 g/kg of DM and ruminal ammonia-N concentration of 0.1 mg/100 mL. However, no further improvements in digestibility were achieved beyond diet CP concentration of 118 g/kg of DM and ruminal ammonia-N concentration of 1.7 mg/100 mL. These observations suggest that diet digestibility is not likely to be compromised unless a diet with very low concentration of CP is offered, whereas rumen ammonia-N concentrations above 1 mg/100 mL seem sufficient for optimal diet digestibility. However, in contrast to these conclusions a previous meta-analysis of 497 diets indicated a consistent positive relationship between diet CP concentration and NDF digestibility (Nousiainen et al., 2009). In that meta-analysis, NDF digestibility increased by 7.7 g/kg per 1-percentage-unit increase in diet CP concentration. In the current study, lack of response in NDF digestibility to urea-N infusions may be due to lack of statistical power in a single experiment because the slope of regression between diet CP concentration and NDF digestibility was 6.6 g/kg per 1-percentage-unit increase in diet CP concentration.

Broderick et al. (2010) concluded, based on a meta-analysis of 32 studies, that the omasal canal nonammonia-N flows were equal to N intake at diet CP concentration of 147 g/kg of DM and ruminal ammonia-N concentration of 7.1 and MUN concentration of 8.3 mg/100 mL. Under those circumstances, and assuming a steady state, there is no net absorption of ammonia-N from the rumen and the amount of CP degraded in the rumen per unit of time equals microbial CP synthesis. Consistent with these estimates, Castillo et al. (2001) noted an exponential increase in urinary N excretion of dairy cows above diet CP concentrations of 140 g/kg of DM. In a summary of several studies, Schwab et al. (2005) concluded that ammonia-N concentrations between 7 to 15 mg/100 mL were needed to maximize microbial N flow from the rumen. Based on in vitro studies, Russell and Strobel (1987) concluded that at least 50 mg of ammonia/L (4.1 mg/100 mL of ammonia-N) was needed to maximize bacterial protein synthesis. In the current study, based on the above-mentioned criteria, ruminal ammonia-N concentrations were suboptimal for the maximum rate of microbial-N synthesis on all urea-N infusion levels. As estimated above, despite low ruminal ammonia-N concentrations, rumen microbes captured approximately 40% of supplementary urea-N, but eventually the major proportion (53%) of supplementary urea-N was excreted in urine.

These observations tend to suggest that even under limiting conditions rumen microbes cannot completely utilize supplementary ammonia-N.

Once absorbed from the gut, ammonia-N will be eliminated in urine unless recycled into the gastrointestinal tract. The efficiency of urea-N recycling and the contribution of recycled urea-N to microbial N synthesis are dependent not only on plasma urea and rumen ammonia concentrations but also on the permeability of gut epithelia that is affected by the N status of the animal (Kennedy and Milligan, 1980; Kristensen et al., 2010). The effects of diet composition on the efficiency of urea-N recycling seem to be associated with adaptive changes in animal physiology. In studies with dry cows, Bailey and Balch (1961) observed a linear relationship between saliva and plasma urea concentrations with saliva concentrations being 65% of those in plasma. Based on a series of experiments in steers employing radionuclide tracer techniques, Kennedy (1980) concluded that urea-N entering the rumen in saliva was dependent on plasma urea concentrations but independent of rumen ammonia-N concentration. In contrast, the direct transfer of plasma urea from capillaries through the rumen wall into the rumen was positively related to plasma urea and negatively to rumen ammonia-N concentrations (Kennedy, 1980). In addition, increases in the rate of rumen fermentation and microbial energy supply seem to enhance the transfer of plasma urea into the gut. Supplementation of forage diets with sucrose increased the rate of plasma entry into the gut of steers by 35% (Kennedy, 1980). Kennedy and Milligan (1980) pointed out an inverse relationship between urea transfer from plasma to the rumen and rumen ammonia-N concentration in sheep and cattle. This negative relationship decreases the contribution of recycled urea-N to microbial N synthesis and partly explains the diminishing returns to supplementation of a diet with protein feeds.

Milk Urea Nitrogen

If diet CP concentration can be readily and reliably determined on farm it is a good predictor of N use efficiency for milk protein synthesis (Huhtanen and Hristov, 2009). Milk urea-N concentration is primarily dependent on diet CP concentration and can be used to predict the N use efficiency, rumen N balance, and urine N excretion in dairy cows (Broderick and Clayton, 1997; Nousiainen et al., 2004). However, Spek et al. (2013) identified several factors influencing the relationship between MUN concentrations and urinary urea-N excretion in dairy cattle. Milk urea-N concentrations are affected by factors such as the time of milk sampling relative to feed intake pattern, water intake

and diet mineral concentrations, feeding and milking frequency, and analytical methods (enzymatic vs. infrared spectrometry).

Considering MUN as a diagnostic tool for an adequate diet CP concentration, it is noteworthy that in the current study MUN responded to increases in supplementary urea in a more consistent manner than the rumen ammonia-N concentrations. In agreement with these observations Broderick and Clayton (1997) reported a closer relationship between MUN and diet CP concentration ($CP\% = 0.269 \times MUN + 13.7$; $R^2 = 0.84$) than between MUN and ruminal ammonia-N (NH_3 , mg of N/100 mL = $0.686 \times MUN + 6.43$; $R^2 = 0.57$). Increases in postruminal N supply provided as casein infused into the abomasum increased plasma urea-N and MUN concentration but did not have effects on rumen ammonia-N concentrations (Vanhatalo et al., 2003). Urea-N concentrations in milk and plasma are closely correlated (Broderick and Clayton, 1997) but also exhibit diurnal variation that reflects ruminal pattern of ammonia-N concentrations with 1.5 to 2 h lag between rumen ammonia-N and blood urea-N and about 1 h lag between blood urea-N and MUN (Gustafsson and Palmquist, 1993). If dairy cow diets are to be formulated solely based on diet CP concentration rather than based on distinct requirements for RDP and RUP there should be a close relationship between diet CP concentration and plasma urea concentration. The slope of the relationship between diet CP concentration and MUN in the current study [$MUN \text{ (mg/100 mL)} = -16.9 + 0.17 \times \text{diet CP (g/kg of DM)}$] was similar to that reported by Nousiainen et al. (2004) based on 50 production trials with 306 treatment means [$MUN \text{ (mg/100 mL)} = -14.2 + 0.17 \times \text{diet CP (g/kg of DM)}$]. A potential explanation for the smaller intercept estimated for the current study than reported by Nousiainen et al. (2004) might be the high digestibility and good fermentation quality of grass silage used in the current study. When cows were offered either early (714 g/kg of DM of digestible OM) or late harvested grass silage (673 g/kg of DM of digestible OM) observed MUN was 0.4 mg/100 mL lower than that predicted based on diet CP concentration for the early harvested and 0.9 mg/100 mL higher than predicted for the late harvested grass silage (Kuoppala et al., 2009, 2010).

CONCLUSIONS

The results from the current study indicate that dairy cows offered a diet slightly deficient with respect to RUP benefit from supplementary RDP provided as urea-N. However, quantitatively the effects were small and cows did not show any acute signs of deficiency

but adapted well to the basal diet despite very low rumen ammonia-N and milk urea-N concentrations. The efficiency of N utilization tended to decrease in a linear manner with increases in supplementary urea-N. Eighteen percent of supplementary urea-N infused into the rumen was secreted in milk, whereas the remainder was excreted in urea and feces. Milk urea-N concentrations responded to increases in intraruminal N supply in a consistent manner, whereas rumen ammonia-N concentrations increased only at the highest level of urea-N infusion.

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