Effect of time duration of ruminal urea infusions on ruminal ammonia concentrations and portal-drained visceral extraction of arterial urea-N in lactating Holstein cows

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ABSTRACT

The effects of a 6 versus 24 h ruminal urea infusion in lactating dairy cows fed a basal diet deficient in N on ruminal ammonia concentration, arterial urea-N concentration, net portal-drained viscera (PDV) urea-N flux, arterial urea-N extraction across the PDV, and renal urea-N kinetics were investigated. Three Danish Holstein cows fitted with ruminal cannulas and permanent indwelling catheters in major splanchnic blood vessels were randomly allocated to a 3 × 3 Latin square design with 21-d periods. Treatments were ventral ruminal infusion of water for 24 h (water INF), 24-h infusion of 15 g of urea/kg of dry matter intake (DMI; 24-h INF), and 6-h infusion of 15 g of urea/kg of DMI (6-h INF). The 6-h INF was initiated 0.5 h after the afternoon feeding, and ran until 2230 h. Eight sample sets of arterial, portal, and hepatic blood, ruminal fluid, and urine were obtained at 0.5 h before the morning feeding and 0.5, 1.5, 2.5, 3.5, 4.5, 5.5, and 6.5 h after feeding (i.e., 9 to 15.5 h after the 6 h infusion was terminated). A substantial decrease in DMI for 6-h INF compared with 24-h INF and water INF was observed, and it has to be recognized that DMI may have confounding effects. However, the experimental setting plan was met (i.e., to cause changes in the daily pattern of ruminal ammonia and blood urea-N concentrations). The arterial urea-N concentration for 24-h INF and 6-h INF were greater than the arterial urea-N concentration with water INF throughout the sampling window. However, the arterial urea-N concentration for 6-h INF decreased steadily with sampling time reflecting a carryover effect from the ruminal urea infusion. The ruminal ammonia concentration and net portal flux of ammonia for 6-h INF were not different from water INF; hence, no carryover effect on ruminal ammonia concentration was observed. The portal flux

of urea-N was not affected by treatment (i.e., even the combination of low ruminal ammonia and high arterial urea-N concentration with 6-h INF was not used by the cow to increase the uptake of urea-N across the PDV). Arterial urea-N extraction across the PDV was increased with water INF especially from 0.5 to 3.5 h postprandial relative to the urea infusion treatments, reflecting increased epithelial permeability for urea-N. This indicates that daily ruminal peak of ammonia or blood urea-N concentrations overruled potential signals from low ruminal ammonia concentration observed during the sampling window. In conclusion, dairy cows appear unable to increase transport of urea-N from blood to gut in periods with low ruminal ammonia concentrations, even in a situation with infrequent N supply and apparent carryover effects on blood urea-N. It is speculated that mechanisms responsible for down-regulation of epithelial urea-N transport based on daily maximum concentrations of ammonia in the rumen or urea-N in the blood suppresses any short-term signal from low ruminal ammonia during periods with low ruminal N supply.

Key words: dairy cow, urea-N recycling, extraction, urea transporter-B mRNA

INTRODUCTION

The ability of the ruminant to recycle urea-N from the blood to the gut might serve an advantage for the animal in situations where N supply is deficient by increasing the availability of ruminal N for anabolic purposes. Infrequent protein supplementation has been used as a strategy for improving N retention and decreasing urinary N excretion. In several studies using this strategy, mainly with growing beef cattle and lambs, only few negative effects on N retention were observed when protein supplementation frequency did not extend 3 d (Reynolds and Kristensen, 2008). It has been suggested that sustained increased arterial urea-N concentration and portal-drained viscera (PDV) uptake of urea-N after supplementation could be responsible for the lack of negative effects of infrequent protein supplementation

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(Krehbiel et al., 1998; Bohnert et al., 2002). In addition, a downregulation of renal urea-N clearance during low-N periods would enhance the recycling of urea-N to the gastrointestinal tract (Marini et al., 2006). However, most of the work done with infrequent protein supplementation for improving N efficiency does not apply to dairy cows and the functional properties of urea-N transport and, hence, the mechanisms regulating urea-N transport across the PDV epithelium are not fully elucidated. In a recent study, arterial urea-N extractions across the PDV and rumen did not change when blood urea-N concentration was increased shortly with primed continuous intravenous urea infusion, implying that urea-N transport was directly proportional to arterial urea-N concentrations, hence regulated by mass action (Kristensen et al., 2010). In contrast, when ruminal infusion of urea was used to increase daily ruminal N supply a postprandial decrease in ruminal extraction of urea-N was observed, cancelling the effect of postprandial increases in arterial blood urea-N concentrations, reflecting a short-term regulatory mechanism affecting ruminal urea-N transport (Røjen et al., 2011b). Additionally, the permeability of the epithelia adapts to changes in dietary status (i.e., long-term effects; Kristensen et al., 2010; Røjen et al., 2011b). This implies a rather complicated regulatory mechanism for urea-N transport. The discovery of urea transporter (UT)-B proteins in the epithelium lining the gut of ruminants (Ritzhaupt et al., 1997; Stewart et al., 2005) offers a possible mechanism for explaining changes in urea permeability under changing dietary N conditions.

Understanding the carryover effects with infrequent protein supplementation on arterial urea-N concentration and urea-N recycling to compensate low ruminal ammonia concentrations might be important tools to increase N efficiency of dairy cattle under various feeding conditions. The purpose this study was to induce different diurnal patterns of N availability in the rumen of lactating dairy cows via 2 N-supplementation treatments differing in the duration of ruminal urea infusion when fed a basal ration with low RDP concentration. The aim was to investigate the N buffering, and how critical short-term fluctuations in N availability is to the daily regulation of urea-N transport in dairy cows. We hypothesized that ruminal ammonia concentration with infrequent N supply would be buffered by urea-N transport from blood to gut during times of no infusion. The study was designed to investigate the effects of a) continuous ruminal infusion of urea (24 h) or b) the same amount of urea in short-term ruminal infusion (6 h) on ruminal ammonia concentration, ammonia absorption, arterial blood urea-N concentration, PDV net flux of urea-N, PDV extraction of arterial urea-N,

and renal urea-N kinetics. In addition, the expression of UT-B mRNA in ruminal papillae was investigated.

MATERIALS AND METHODS

The present experiment complied with the Danish Ministry of Justice, Law no. 382 (June 10, 1987), Act no. 726 (September 9, 1993) concerning experiments with animals and care of experimental animals.

Animals and Experimental Design

Three Danish Holstein cows in their second lactation were used. Cows were fitted with a ruminal cannula (#1C; Bar Diamond Inc., Parma, ID) and permanent indwelling catheters in an artery, mesenteric vein, hepatic portal vein, hepatic vein, and gastrosplenic vein as described previously (Larsen and Kristensen, 2009; Røjen et al., 2011b). Cows were kept in tie-stalls with rubber mats and wood shavings as bedding. Cows were milked at 0530 and 1530 h, and milk production was recorded at each milking. The first samplings in the present study were at 120 ± 4 DIM. Cows were randomly allocated to a 3×3 Latin square with 21-d periods. Treatments were continuous ventral ruminal infusion of urea for 24 h (**24-h INF**) or short-term ventral urea infusion for 6 h (**6-h INF**). The planned level of urea for infusion was 15 g/kg of DMI. As a negative control, tap water (**water INF**, 10 L/d) was infused continuously into the ventral rumen. With the 6 h infusion treatment, experimental sampling was done 9 to 15.5 h after the infusion was stopped. All cows were fed the same basal TMR (Table 1). The RDP supply of basal TMR was estimated to be 19 g/kg of DM below minimum requirements (NorFor, 2011) and, consequently, supplied only 75% of requirements for MP for cows producing 25 kg of ECM/d with a DMI of 18 kg/d. The basal TMR adequately supplied all other nutrients. Cows were fed in 3 equal portions at 0800, 1600, and 2400 h to allow for an 8-h sampling window and refusals were removed and weighed at 0730 h. The first 14 d of each experimental period the basal diet was fed to obtain approximately 5% feed refusals and voluntary DMI for each cow was determined as the average DMI during this period. Each cow was fed at 95% of her voluntary DMI for the remainder of the experimental period to ensure constant daily intake to obtain the predefined urea infusion relative to DMI. Cows had access to salt mineral blocks (Salto Universal; Vitfoss, Gråsten, Denmark).

The urea infusion treatments were designed to provide 15 g of urea/kg of DMI. The infusates were prepared daily and the amount of feed urea (SKW Stickstoff-

Table 1. Ingredient and nutrient composition of the basal diet (g/kg of DM if not otherwise noted)

Item	Experimental diet
Ingredient	
Corn silage ¹	436
Clover grass silage ²	185
Barley, rolled	250
Rapeseed cake, 13% fat	24
Molasses, sugar beet	10
Sugar beet pulp, dried	64
Mineral premix ³	10
NaHCO ₃	7.5
Na ₂ SO ₄	2.5
Vegetable fat ⁴	7.5
HMBi ⁵	2.6
Chromium(III)oxide	1
Nutrient	
DM, g/kg	460 ± 6.0
Ash	57 ± 2.6
NDF	307 ± 8.5
Crude fat	38 ± 0.2
CP	114 ± 1.0
Calculated nutrient composition	
RDP ⁶	79
MP (NorFor AAT) ⁶	77
NE _L , ⁶ MJ/kg of DM	6.81

¹Chemical composition of corn silage: DM, 363 (g/kg); ash, 31; CP, 87; NDF, 375; starch, 320; in vitro OM digestibility, 72.5%.

²Chemical composition of grass clover silage: DM, 407 (g/kg); ash, 86; CP, 146; NDF, 363; in vitro OM digestibility, 78.8%.

³Mineral premix (VM 2; Vitfoss, Gråsten, Denmark) contained (per kilogram): 160 g of Ca, 45 g of P, 60 g of Mg, 80 g of Na, 5 g of S, 546 kIU of vitamin A, 173 kIU of vitamin D, 3,640 mg of α -tocopherol, 3,640 mg of Mn, 820 mg of Cu, 23 mg of Co, 4,090 mg of Zn, 204 mg of I, and 45 mg of Se.

⁴Palm oil fatty acid distillate.

⁵HMBi 2-hydroxy-4-(methylthio)-butanoic acid isopropyl ester (MetaSmart; Adisseo S.A.S., Antony, France).

⁶Calculated using the Nordic Feed Evaluation System (NorFor, 2011).

werke Piesteritz GMBH, Lutherstadt Wittenberg, Germany) for infusion was calculated each day according to DMI the previous 24 h. Infusions began on the first day of each experimental period and ran until the end of an actual sampling day, which was on the last day of each period. Water INF and 24-h INF treatments were continuous throughout the day except for about 20 min in the afternoon where infusates were refilled. The 6-h INF was initiated 0.5 h after the afternoon feeding at 1630 h and ran until 2230 h. Water and feed urea were added to 10-L Jerry cans and mixed to ensure that feed urea was completely dispersed into water. Adjustable-speed 2-channel peristaltic pumps (Type 115; Ole Dich Instrumentmakers ApS, Hvidovre, Denmark) were used for the infusions through silicone tubing (3 mm i.d., 6 mm o.d.; Ole Dich Instrumentmakers) at a rate of 414 ± 30 g/h. Infusion lines were inserted through the ruminal cannula and a weight fastened at the tip of the tubing was used to help anchor the infusion tub-

ing in the ventral ruminal sac. Placement was checked regularly. Several small holes were made 18 to 8 cm from the tip of the tubing to allow diffusion into rumen content.

Experimental Samplings

Blood flows were determined by downstream dilution of *p*-aminohippuric acid (pAH) according to Katz and Bergman (1969). Sterile pAH (175 mmol/kg) was infused continuously into the mesenteric vein at 28 ± 3 mmol/h. The infusion was initiated at 0530 h, 2 h before first urine sampling to ensure that urine concentration of pAH had reached steady state at first sampling. Quasi steady-state conditions for pAH in blood were ensured by visual inspection of plots of plasma pAH concentrations versus time. Eight sets of blood, ruminal fluid, and urine samples were obtained at 0.5 h before feeding and at 0.5, 1.5, 2.5, 3.5, 4.5, 5.5, and 6.5 h after 0800 h feeding. Before blood sampling, catheters were primed by drawing a minimum of 2 times the catheter volume into a blank syringe and the blood discarded. Blood was sampled simultaneously from the artery, hepatic portal vein, and hepatic vein by slowly drawing blood into 20-mL syringes. Blood was transferred into sodium heparin Vacuettes (Greiner Bio-One GmbH, Kremsmünster, Austria) and K₃EDTA Vacuettes (Greiner Bio-One GmbH), mixed and placed on ice immediately after collection. Separate blood samples were obtained in 1-mL heparinized syringes and immediately taken for blood gas measurements. Plasma was harvested after centrifugation at $3,000 \times g$ at 4°C for 20 min and stored at -20°C until analysis. Ruminal fluid was sampled from the ventral ruminal sac using an extended suction strainer (Bar Diamond Inc.) and a 60-mL syringe. A subsample of ruminal fluid was stabilized with 25% meta-phosphoric acid and stored at -20°C. Eight samples of urine were collected at the same time points as blood sampling by stimulating the cow to urinate in a cup by sweeping the supra mammary region by hand and pH was measured immediately. Subsamples were stored at -20°C.

At 1100 to 1200 h, ruminal mucosa from the lateral face of the cranioventral sac (atrium) was biopsied through the rumen cannula by snapping off 15 to 20 papillae by hand, and papillae were submerged in RNA stabilization solution (RNAlater; Sigma-Aldrich Inc., St. Louis, MO) in 2.0-mL Eppendorf tubes, stored at 4°C overnight, and then transferred to -80°C for urea transporter mRNA expression. Samples for measurement of milk constituents were collected on sampling days. Feed samples from the last 7 d of each experimental period were pooled for chemical analysis. Cows were

weighed before the afternoon milking of each sampling day.

Analytical Procedures

Feed samples were dried at 60°C for 48 h in a forced-air oven for DM determination. Feed samples from the last 7 d of each experimental period were pooled for chemical analysis. Organic matter was determined as DM – crude ash, where crude ash content was determined after combustion at 525°C for 6 h. Crude fat was determined as petroleum ether extract following acid hydrolysis (Stoldt, 1952). Neutral detergent fiber content was determined as described by Mertens (2002). Crude protein was determined as $N \times 6.25$ and N determined by the Dumas method as described by Hansen (1989). Packed cell volume was determined for all arterial samples by centrifugation of micro-capillary tubes at $13,000 \times g$ for 6 min at ambient temperature. Blood collected in 1-mL syringes was immediately taken for blood gas and oximetry analysis (ABL 520; Radiometer A/S, Copenhagen, Denmark). Plasma and rumen fluid concentrations of D-glucose and L-lactate were determined by immobilized oxidase enzyme membranes and ion-selective electrodes using a Select Biochemistry Analyzer (YSI 7100; YSI Inc., Yellow Springs, OH). Plasma and urine concentrations of pAH were determined by the method described by Kristensen et al. (2009) using a continuous flow analyzer (AutoAnalyzer 3; method US-216–17 rev.1; SEAL Analytical Ltd., Burgess Hill, UK). Plasma, urine, and milk urea-N concentrations were determined using the monoxime diacetyl method (Marsh et al., 1965) using a continuous flow analyzer. Before analysis, milk was deproteinized by combining raw milk samples with an equal volume of 24% trichloroacetic acid. Concentrations of ammonia, BHBA, and NEFA were determined in plasma using a Cobas Mira autoanalyzer (Triolab A/S, Brøndby, Denmark) and kits based on glutamate dehydrogenase (AM 1015; Randox Laboratories Ltd., Crumlin, UK), D-3-hydroxybutyrate dehydrogenase (Ranbut; Randox Laboratories Ltd.), and the acyl coenzyme A synthetase/acyl coenzyme A oxidase method (FA 115; Randox Laboratories Ltd.), respectively. Arterial plasma and urine (1:20 dilution) concentrations of creatinine-N were determined using a Cobas Mira autoanalyzer and a kit based on reaction with alkaline picrate (Creatinine 120 CP; Horiba ABX, Montpellier, France). Ammonia in ruminal fluid was determined using a Cobas Mira autoanalyzer after 1:20 dilution with 100 mM phosphate buffer (AM 1015; Randox Laboratories Ltd.). Ruminal fluid with 5% meta-phosphoric acid was analyzed for VFA by gas chromatography (Kristensen et al., 1996). Ruminal fluid pH was measured immediately after sampling using a combination

electrode (PHC2002–8; Hach Lange APS, Brønshøj, Denmark) and a pH meter calibrated at pH 4.005 and 7.000 (PHM 240; Hach Lange APS). Total N in urine was determined as described by Hansen (1989). Urine was analyzed for hippuric acid and 6 amino acids (alanine, glycine, leucine, isoleucine, proline, and valine) by GC-MS according to Kristensen (2000). This method was modified by using adipic acid as internal standard for hippuric acid determination and for amino acid-N determination (Kristensen et al., 2009). Milk was analyzed for the content of protein, fat, and lactose by infrared spectroscopy using a Milkoscan 4000 (Foss Electric A/S, Hillerød, Denmark). Urine concentrations of allantoin-N and uric acid-N were determined by HPLC according to Thode (1999). Quantification of UT-B mRNA expression in rumen papillae was carried out by real-time reverse transcriptase (RT)-PCR. The procedure, and primers used were described by Røjen et al. (2011b).

Calculations and Statistics

Whole blood concentrations of ammonia were set equal to blood plasma concentrations. The whole-blood concentration of urea-N was obtained by correcting for the urea-N dilution space in erythrocytes of 80% (Røjen et al., 2011b). Calculations of net portal, net hepatic, and total splanchnic metabolite fluxes, and PDV extraction ratio of urea-N were performed as described by Røjen et al. (2008). A positive net flux indicates a net release from a tissue to the blood whereas a negative net flux indicates a net uptake by the tissue. Renal urea-N clearance (volume of blood urea-N cleared by the kidneys per time unit), filtered load of urea-N (urea-N filtered at glomerulus), urea-N reabsorbed (percentage of filtered load of urea-N not excreted), and other renal kinetic variables were calculated according to Røjen et al. (2011b). Quantification of mRNA expression of UT-B was performed as described by Theil et al. (2006), and Røjen et al. (2011b). Briefly, data was obtained as cycle threshold (Ct) values (the number of PCR cycles required to reach a certain threshold). Least squares means of ΔCt values ($\Delta Ct = Ct$ of the target gene – Ct of the housekeeping gene) were normalized to water INF by calculating the $\Delta\Delta Ct$ values (ΔCt observed at given treatment – ΔCt observed with water INF) and the relative mRNA expression was calculated as $E^{-\Delta\Delta Ct}$, where E is $1 + \text{PCR efficiency}$ as determined by $10^{-1/\text{slope of standard curve}} - 1$. All statistics were performed at the ΔCt level to exclude potential bias because of averaging data that had been transformed through the equation $E^{-\Delta\Delta Ct}$.

Data on blood, ruminal, and urine variables were analyzed using the MIXED procedure of SAS [Statisti-

cal Analysis System version 9.1 (TS1M3); SAS Institute Inc., Cary, NC] according to a 3×3 Latin square design with treatment, period, sampling time, and the interaction treatment $\times$ sampling time as fixed effects. Cow was considered a random effect. Samplings within cow and period were considered as repeated measures. There were no missing observations. Covariance structures were modeled using first-order factor analytics, heterogeneous autoregressive first order, and autoregressive first order based on model convergence and fit statistics. Variables with only 1 observation within cow and sampling day were analyzed using a reduced model, not including the effect of sampling time. Data on DMI and milk yield were analyzed using data from the last 4 d before the sampling day. Data are reported as least squares means with residual standard errors of the mean. Significance level was defined at $P \leq 0.05$ and for main effects, tendencies were considered at $0.05 < P \leq 0.10$.

RESULTS

Diet Composition, Feed Intake, and Milk Yield

The percentage of CP of the basal diet was low, but was increased to a moderate level with the treatment infusions (Table 2). Dry matter intake decreased ($P = 0.05$; Table 2) in 6-h INF compared with 24-h INF and water INF (10.6, 18.0, and 17.4 kg of DMI/d, respectively), which resulted in a calculated negative energy balance with 6-h INF of 29 MJ/d. The amount of urea infused was designed to be 15 g/kg of DMI and initially the study was designed for similar feed intake between treatments, providing the same amount of urea with 6-h INF and 24-h INF. However, the amount of urea infused was adjusted each day and calculated according to daily DMI, and due to the observed decrease in DMI with 6-h INF, the infusion rate was higher in 24-h INF compared with 6-h INF (361 and 242 ± 29 mmol/h; data not shown). The CP digestibility tended ($P = 0.08$) to increase for urea-N infusions compared with water infusion. The CP digestibility did not differ ($P = 0.68$) between treatments when the CP supply from urea infusions were assumed 100% degradable. Total tract digestibility of NDF and OM was not affected by treatment ($P \geq 0.23$). Milk yield, ECM, protein, milk N yield, and lactose yield decreased ($P \leq 0.05$) in 6-h INF compared with 24-h INF and water INF. Milk fat yield tended ($P = 0.09$) to decrease with 6-h INF. The milk composition of fat and protein were not affected ($P \geq 0.16$) by treatment, whereas lactose concentration tended to decrease with 6-h INF. The urea-N concentration in the morning milk tended to be lower in water INF compared with 24-h INF and 6-h INF ($P = 0.06$).

At the evening milking the milk urea-N concentration was greater ($P = 0.04$) in 24-h INF compared with water INF. The calculated apparent N utilized for milk protein was not affected ($P = 0.12$) by treatment.

Ruminal Variables

Ruminal pH, concentration of glucose and L-lactate were not affected ($P \geq 0.55$; Table 3) by treatment. A treatment by sampling time interaction ($P = 0.03$) for the concentration of ammonia in the ventral ruminal fluid was observed. The concentration of ammonia was greater in 24-h INF compared with water INF and 6-h INF (Figure 1). Generally, ammonia concentrations increased immediately after feeding, with the zenith at 0.5 to 1.5 h after feeding, and then decreased toward prefeeding levels. The postprandial increase was more pronounced with 24-h INF.

The total concentration of ruminal VFA increased ($P = 0.02$) in 24-h INF compared with water INF and 6-h INF. Ruminal pH, and concentrations of ammonia and L-lactate were affected ($P < 0.01$) by sampling time. A postprandial decrease in ruminal pH was followed by an increase toward the end of the sampling window. Postprandial increase in L-lactate concentration was observed, with the zenith at 0.5 h and decreasing quickly thereafter (data not shown). The molar proportions of ruminal VFA were not affected ($P \geq 0.64$) by treatment, except for the molar proportion of isobutyrate, which was smaller in 24-h INF and greater in 6-h INF ($P < 0.01$) compared with water INF. The molar proportion of acetate decreased after feeding and was greater (treatment by sampling time interaction $P = 0.05$) for 6-h INF 4.5 to 6.5 h after feeding compared with water and 24-h INF (data not shown). The molar proportion of isovalerate, valerate, and caproate increased ($P \leq 0.02$) after feeding. The ruminal concentration of glucose and total VFA, and the molar proportions of propionate, isobutyrate, and butyrate were not affected ($P \geq 0.11$) by sampling time.

Arterial Variables

Arterial blood pH, arterial blood concentrations of ammonia and carbon dioxide, and blood plasma concentrations of pAH, glucose, and BHBA were not affected ($P \geq 0.28$; Table 4) by treatment. Treatment by sampling time interactions for arterial blood pH and BHBA were observed ($P \leq 0.02$). The plasma concentration of BHBA with 6-h INF and 24-h INF increased from 0.5 to 1.5 h after feeding, followed by a decrease, whereas with water INF, a slow postprandial increase was observed at 3.5 h and remained steady thereafter (data not shown). No meaningful interpretation of the

Table 2. Effect of ruminal urea infusion on DMI, nutrient digestibility, milk yield, milk composition, apparent N utilization for milk protein, and BW in lactating dairy cows

Item	Treatment ¹			SEM ²	P-value
	Water INF	24-h INF	6-h INF		
CP, % of DM	11.43	15.63	16.22	—	—
DMI, kg/d	17.4 ^b	18.0 ^b	10.6 ^a	1.25	0.05
Total N supply, g/d	317 ^a	451 ^b	275 ^a	29.0	0.03
Energy balance, ³ MJ/d	1	−1	−29	—	—
Nutrient apparent digestibility					
CP, with urea from infusions, %	58	69	68	2.0	0.08
CP, with urea assumed to be 100% degradable, %	58	58	55	2.3	0.68
NDF, %	52	59	54	2.4	0.34
OM, %	74	77	74	1.0	0.23
Yield					
Milk, kg/d	24.8 ^b	27.1 ^b	20.5 ^a	0.83	0.05
ECM, ⁴ kg/d	24.7 ^b	26.6 ^b	20.4 ^a	1.01	0.05
Milk fat, kg/d	1.03	1.11	0.89	0.067	0.09
Milk protein, kg/d	0.75 ^b	0.80 ^b	0.58 ^a	0.017	0.02
Milk lactose, kg/d	1.22 ^b	1.34 ^b	0.99 ^a	0.035	0.04
Milk composition					
Fat, g/kg	41.6	41.0	43.9	3.32	0.22
Protein, g/kg	30.3	29.5	28.5	0.65	0.16
Lactose, g/kg	49.4	49.6	48.4	0.62	0.06
Urea-N in a.m. milk, mM	2.78	9.57	11.19	1.108	0.06
Urea-N in p.m. milk, mM	3.22 ^a	9.27 ^b	5.85 ^{ab}	0.592	0.04
Apparent N utilization for milk protein, %	37	28	34	2.6	0.12
BW, kg	568	562	527	12.0	0.06

^{a,b}Means within a row with different superscripts differ ($P \leq 0.05$).

¹Treatments were 24-h ruminal infusion of water (water INF), 24-h ruminal infusion of 15 g of feed urea/kg of DMI (24-h INF), and 6-h ruminal infusion of 15 g of feed urea/kg of DMI (6-h INF). Cows were sampled 9 to 15.5 h after termination of 6-h infusion.

² $n = 3$.

³Calculated using the Nordic Feed Evaluation System (NorFor, 2011).

⁴Calculated according to Sjaunja et al. (1991).

treatment by sampling time interaction for arterial blood pH can be given. The hematocrit of the blood, the blood concentration of oxygen, and the plasma concentration of NEFA increased ($P \leq 0.03$) in 6-h INF compared with water INF and 24-h INF. For arterial blood concentration of urea-N a treatment by sampling

time interaction ($P = 0.03$) was observed. Arterial blood concentration of urea-N for 6-h INF decreased steadily with time, whereas the concentrations of urea-N with water INF and 24-h INF remained relatively stable during sampling times with only a small postprandial increase (Figure 2). The blood concentration

Table 3. Effect of ruminal urea infusion on ruminal variables in lactating dairy cows

Item	Treatment ¹				P-value ³		
	Water INF	24-h INF	6-h INF	SEM ²	Trt	Time	Trt × Time
Ruminal pH	6.46	6.32	6.33	0.137	0.66	<0.01	0.19
Ammonia, mmol/L	2.90 ^a	9.11 ^b	3.44 ^a	0.546	<0.01	<0.01	0.03
Glucose, mmol/L	0.09	0.10	0.10	0.007	0.55	0.18	0.23
L-Lactate, mmol/L	0.74	1.10	0.52	0.479	0.56	<0.01	0.93
Total VFA, mmol/L	100 ^a	112 ^b	101 ^a	2.8	0.02	0.35	0.30
Acetate, mol/100 mol	58.4	58.5	59.1	1.36	0.72	0.01	0.05
Propionate, mol/100 mol	23.6	23.3	22.3	2.25	0.89	0.38	0.37
Isobutyrate, mol/100 mol	0.77 ^b	0.68 ^a	0.82 ^c	0.019	<0.01	0.11	0.06
Butyrate, mol/100 mol	13.0	13.5	13.5	1.15	0.94	0.16	0.38
Isovalerate, mol/100 mol	1.60	1.60	1.70	0.085	0.64	<0.01	0.32
Valerate, mol/100 mol	1.67	1.63	1.58	0.080	0.78	0.02	0.08
Caproate, mol/100 mol	0.88	0.82	0.95	0.169	0.87	0.02	0.26

^{a-c}Means within a row with different superscripts differ ($P \leq 0.05$).

¹Treatments were 24-h ruminal infusion of water (water INF), 24-h ruminal infusion of 15 g of feed urea/kg of DMI (24-h INF), and 6-h ruminal infusion of 15 g of feed urea/kg of DMI (6-h INF). Cows were sampled 9 to 15.5 h after termination of 6-h infusion.

² $n = 3$.

³P-values for Trt = treatment, time within sampling day (Time), and Trt × Time interaction effects.

Figure 1. Ruminal ammonia concentrations (mmol/L) relative to time of feeding in lactating Holstein cows. Treatments were 24-h ruminal infusion of water (water INF; circles), 24-h ruminal infusion of 15 g of feed urea/kg of DMI (24-h INF; squares), and 6-h ruminal infusion of 15 g of feed urea/kg of DMI (6-h INF; triangles). Cows were sampled 9 to 15.5 h after termination of 6-h infusion. A treatment by sampling time interaction ($P = 0.03$) was observed, reflecting a greater ruminal concentration of ammonia in 24-h INF compared with water INF and 6-h INF, and the postprandial increase being more pronounced with 24-h INF. The arrow indicates time of feeding. Data points represent the means of 3 cows $\pm$ standard errors of the means.

of urea-N was less ($P = 0.03$) in water INF compared with 6-h INF and 24-h INF. The plasma concentration of L-lactate decreased ($P < 0.01$) in water INF compared with 6-h INF and 24-h INF. Arterial blood pH, arterial blood concentrations of oxygen, and plasma concentrations of pAH, glucose, and L-lactate were not

affected ($P \geq 0.34$) by sampling time. The arterial hematocrit, arterial blood concentration of ammonia, and the plasma concentration of NEFA and BHBA were affected ($P \leq 0.05$) by sampling time, but the time effect for arterial hematocrit was difficult to interpret. For arterial blood concentration of ammonia, a slight postprandial increase was observed, followed by a decrease toward prefeeding levels, whereas arterial plasma concentration of NEFA decreased after feeding. The arterial blood concentration of carbon dioxide tended ($P = 0.10$) to be affected by sampling time, reflected by a slight postprandial decrease from 1.5 to 2.5 h after feeding (data not shown).

Blood Flows and Net Fluxes

The portal and hepatic whole-blood flows decreased ($P \leq 0.01$; Table 5) in 6-h INF compared with water INF and 24-h INF, whereas hepatic arterial whole-blood flow was not affected ($P = 0.77$) by treatment. The portal, hepatic, and hepatic arterial whole-blood flows were not affected ($P \geq 0.50$) by sampling time.

The net portal blood flux of urea-N, carbon dioxide, and the net portal plasma flux of glucose, BHBA, and L-lactate were not affected ($P \geq 0.26$; Table 5) by treatment. The net absorption of ammonia to the portal blood (positive net portal flux) increased ($P < 0.01$) in 24-h INF compared with water INF and 6-h INF (Figure 3). The ammonia absorption was not different between 6-h INF and water INF during the sampling window. The net portal blood flux of oxygen tended ($P = 0.07$) to decrease in 6-h INF compared with 24-h INF. Net absorption of ammonia was affected ($P <$

Table 4. Effect of ruminal urea infusion on arterial variables in lactating dairy cows

Item	Treatment ¹				P-value ³		
	Water INF	24-h INF	6-h INF	SEM ²	Trt	Time	Trt $\times$ Time
Whole blood							
pH	7.43	7.43	7.42	0.007	0.89	0.56	0.02
Hematocrit, %	23.4 ^a	23.4 ^a	25.5 ^b	1.02	<0.01	0.05	0.45
Ammonia, mmol/L	0.065	0.072	0.070	0.009	0.84	0.04	0.48
Urea-N, mmol/L	2.97 ^a	9.63 ^b	7.65 ^b	1.623	0.03	0.03	0.02
Oxygen, mmol/L	4.47 ^a	4.45 ^a	4.92 ^b	0.180	<0.01	0.11	0.52
Carbon dioxide, mmol/L	25.3	24.9	23.4	0.81	0.28	0.10	0.27
Blood plasma							
p-Aminohippuric acid, mmol/L	0.087	0.074	0.097	0.011	0.29	0.48	0.06
Glucose, mmol/L	3.67	3.87	3.77	0.118	0.38	0.44	0.43
L-Lactate, mmol/L	0.43 ^b	0.45 ^b	0.34 ^a	0.039	<0.01	0.34	0.64
NEFA, mmol/L	0.052 ^a	0.055 ^a	0.096 ^b	0.012	0.03	<0.01	0.06
BHBA, mmol/L	0.55	0.64	0.67	0.130	0.76	0.02	<0.01

^{a,b}Means within a row with different superscripts differ ($P \leq 0.05$).

¹Treatments were 24-h ruminal infusion of water (water INF), 24-h ruminal infusion of 15 g of feed urea/kg of DMI (24-h INF), and 6-h ruminal infusion of 15 g of feed urea/kg of DMI (6-h INF). Cows were sampled 9 to 15.5 h after termination of 6-h infusion.

²n = 3.

³P-values for Trt = treatment, time within sampling day (Time), and Trt $\times$ Time interaction effects.

Figure 2. Arterial blood urea-N concentrations (mmol/L) relative to time of feeding in lactating Holstein cows. Treatments were 24-h ruminal infusion of water (water INF; circles), 24-h ruminal infusion of 15 g of feed urea/kg of DMI (24-h INF; squares), and 6-h ruminal infusion of 15 g of feed urea/kg of DMI (6-h INF; triangles). Cows were sampled 9 to 15.5 h after termination of 6-h infusion. A treatment by sampling time interaction ($P = 0.02$) was observed, reflecting arterial blood concentration of urea-N for 6-h INF decreasing steadily with time, whereas the concentrations of urea-N with water INF and 24-h INF remained relatively stable during sampling times with only small postprandial increases. The arrow indicates time of feeding. Data points represent the means of 3 cows $\pm$ standard errors of the means.

0.01; Figure 3) by sampling time, reflected by a postprandial increase with the zenith 0.5 to 1.5 h after feeding and followed by a decrease toward the prefeeding level. All other variables were not affected ($P \geq 0.14$) by sampling time.

The net hepatic release of blood urea-N, the net hepatic uptake (negative net hepatic flux) of blood ammonia, and the net hepatic release of plasma glucose increased ($P \leq 0.03$; Table 5) in 24-h INF compared with water INF and 6-h INF. The net hepatic blood fluxes of oxygen and carbon dioxide, and net hepatic plasma fluxes of L-lactate were not affected ($P \geq 0.34$) by treatment. A treatment by sampling time interaction for net hepatic blood flux of BHBA ($P < 0.01$) was observed; however, no interpretable pattern was apparent. The net hepatic blood flux of urea-N, ammonia, oxygen, and carbon dioxide, and net hepatic plasma flux of glucose were not affected ($P \geq 0.11$) by sampling time. The net hepatic plasma flux of L-lactate tended to be affected ($P = 0.10$) by sampling time but no meaningful interpretation of this tendency can be given.

The net splanchnic release of urea-N and net splanchnic uptake of oxygen increased ($P < 0.01$; Table 5) in 24-h INF compared with water INF and 6-h INF. A treatment by sampling time interaction for net splanchnic blood flux of ammonia ($P < 0.01$) was observed; however, no interpretable pattern was apparent. Also, a

treatment by sampling time interaction for net splanchnic plasma flux of BHBA ($P = 0.05$) was observed, reflecting a greater postprandial flux in 24-h INF compared with water and 6-h INF. No other splanchnic flux variables were affected ($P \geq 0.26$) by treatment. The net splanchnic blood flux of urea-N, oxygen, and carbon dioxide, and net splanchnic plasma flux of glucose and L-lactate were not affected ($P \geq 0.18$) by time. The net splanchnic blood flux of ammonia was affected ($P < 0.01$) by sampling time, reflecting a less negative flux 0.5 to 2.5 h after feeding (data not shown).

PDV Extractions and UT-B mRNA Expression

A greater PDV extraction of arterial urea-N was observed from 0.5 to 3.5 h after feeding in water INF (treatment by sampling time interaction, $P = 0.03$; Table 5; Figure 4) compared with 6-h and 24-h INF. From 4.5 to 6.5 h after feeding, the extractions were more similar among treatments. The expression of UT-B mRNA in ruminal papillae was not affected ($P = 0.67$) by treatment.

Renal Variables

Urinary pH, plasma concentration of creatinine-N, diuresis, and creatinine-N clearance were not affected ($P \geq 0.11$; Table 6) by treatments. Concentration of creatinine-N in the urine increased ($P < 0.01$) in 6-h INF compared with water and 24-h INF. The renal plasma flow increased ($P = 0.02$) in 24-h INF compared with water and 6-h INF. Urea-N clearance and filtered load of urea-N decreased and urea-N reabsorbed by the kidneys increased ($P \leq 0.03$) in water INF compared with 6-h and 24-h INF. A treatment by sampling time effect ($P = 0.04$) for filtered load of urea-N by the kidney followed the blood urea-N pattern (data not shown). Urinary pH, plasma concentration of creatinine-N, and renal plasma flow were not affected ($P \geq 0.11$) by sampling time. A tendency ($P = 0.10$) for a postprandial increase in urinary creatinine-N concentration was observed, whereas diuresis tended ($P = 0.08$) to decrease throughout the sampling window. A postprandial decrease ($P \leq 0.02$) in renal clearance of creatinine-N and urea-N was observed. The filtered load of urea-N decreased with 6-h INF similar to that observed with arterial blood urea-N concentration (data not shown). Urea-N reabsorbed was also affected by sampling time ($P = 0.01$) but the pattern was difficult to interpret.

For N constituents in urine, excretion of total N and urea-N decreased ($P < 0.01$; Table 6) in water INF compared with 6-h and 24-h INF. Hippuric acid-N excreted in urine increased ($P < 0.01$) in 24-h INF compared with water and 6-h INF. Allantoin-N excreted in

Table 5. Effect of ruminal urea infusion on blood plasma flows, net fluxes of metabolites, and portal-drained visceral (PDV) extraction ratio of arterial urea-N in lactating dairy cows

Item	Treatment ¹				P-value ³		
	Water INF	24-h INF	6-h INF	SEM ²	Trt	Time	Trt × Time
Whole blood flows, ⁴ L/h							
Portal vein	1,532 ^b	1,596 ^b	1,202 ^a	80.1	0.01	0.85	0.47
Hepatic vein	1,840 ^b	1,961 ^b	1,496 ^a	139.2	<0.01	0.80	0.70
Hepatic artery	308	364	294	108.0	0.77	0.50	0.72
Net portal blood flux, ⁵ mmol/h							
Urea-N	-228	-368	-241	37.0	0.31	0.56	0.50
Ammonia	257 ^a	525 ^b	252 ^a	26.5	<0.01	<0.01	0.14
Oxygen	-1,587	-1,699	-1,417	76.7	0.07	0.45	0.51
Carbon dioxide	2,040	1,887	1,466	339.7	0.42	0.81	0.90
Net portal plasma flux, ⁵ mmol/h							
Glucose	45	14	51	26.4	0.54	0.38	0.14
BHBA	152	172	150	26.2	0.73	0.14	0.10
L-Lactate	119	119	98	11.4	0.26	0.26	0.10
Net hepatic blood flux, ⁶ mmol/h							
Urea-N	303 ^a	663 ^b	301 ^a	45.9	<0.01	0.25	0.28
Ammonia	-265 ^a	-535 ^b	-253 ^a	14.7	<0.01	0.11	0.57
Oxygen	-2,077	-2,521	-1,998	239.5	0.34	0.75	0.68
Carbon dioxide	1,511	1,557	1,804	239.0	0.57	0.26	0.93
Net hepatic plasma flux, ⁶ mmol/h							
Glucose	430 ^a	614 ^b	387 ^a	62.2	0.03	0.70	0.34
BHBA	176	239	188	36.5	0.45	0.02	0.01
L-Lactate	-180	-185	-183	28.9	0.95	0.10	0.27
Net splanchnic blood flux, ⁷ mmol/h							
Urea-N	75 ^a	292 ^b	60 ^a	38.3	<0.01	0.18	0.52
Ammonia	-8	-9	-1	6.7	0.67	<0.01	<0.01
Oxygen	-3,664 ^a	-4,220 ^b	-3,414 ^a	257.1	<0.01	0.98	0.88
Carbon dioxide	3,551	3,444	3,270	352.3	0.82	0.90	0.89
Net splanchnic plasma flux, ⁷ mmol/h							
Glucose	476	628	438	101.0	0.26	0.46	0.69
BHBA	328	411	338	102.7	0.81	0.07	0.05
L-Lactate	-62	-66	-84	49.8	0.93	0.23	0.10
PDV extraction of arterial urea-N, ⁸ %	5.11	2.39	2.81	0.32	0.21	0.24	0.03
UT-B mRNA expression ⁹	1.00	0.85	1.17	—	0.67	—	—
	[0.53; 1.88]	[1.07; 1.28]	[0.10; 7.48]				

^{a,b}Means within a row with different superscripts differ ($P \leq 0.05$).

¹Treatments were 24-h ruminal infusion of water (water INF), 24-h ruminal infusion of 15 g of feed urea/kg of DMI (24-h INF), and 6-h ruminal infusion of 15 g of feed urea/kg of DMI (6-h INF). Cows were sampled 9 to 15.5 h after termination of 6-h infusion.

² $n = 3$.

³P-values for Trt = treatment, time within sampling day (Time), and Trt × Time interaction effects.

⁴Blood plasma flows can be obtained by correcting for hematocrit (Table 4): whole blood flow × $[1 - (\text{hematocrit}/100)]$.

⁵Net portal flux, mmol/h = portal blood flow or plasma flow × (portal concentration – arterial concentration), using either whole blood or plasma as indicated.

⁶Net hepatic flux, mmol/h = hepatic blood flow or plasma flow × hepatic concentration – [(portal blood flow or plasma flow × portal concentration) + (hepatic blood flow or plasma flow – portal blood flow or plasma flow) × arterial concentration].

⁷Net splanchnic flux, mmol/h = hepatic blood flow or plasma flow × (hepatic concentration – arterial concentration).

⁸PDV extraction of arterial urea-N, % = [(arterial blood concentration of urea-N – portal concentration of urea-N)/arterial blood concentration of urea-N] × 100.

⁹UT-B = urea transporter-B. Results are relative to the water INF treatment. Lower and upper 95% confidence limits are given in brackets.

urine increased ($P = 0.04$) in 24-h INF compared with 6-h INF. Amino acid-N excreted in urine increased ($P < 0.01$) in 24-h INF compared with water and 6-h INF. Additionally, amino acid-N increased in water INF compared with 6-h INF. Creatinine-N and uric acid-N excretion in urine and N not accounted for in the urine were not affected ($P \geq 0.14$) by treatment. Urinary urea-N excretion was affected ($P < 0.01$) by sampling time similarly to arterial blood urea-N concentration. Creatinine-N excreted in urine increased ($P < 0.01$)

and allantoin-N decreased ($P = 0.03$) with sampling time. Excretion rates of hippuric acid-N and amino acid-N were not affected ($P \geq 0.59$) by sampling time.

DISCUSSION

Effects of Urea Infusion on DMI and Energy Homeostasis

The large decrease in DMI with 6-h INF points toward the applied urea infusion rate being toxic to the

Figure 3. Net portal flux of ammonia (mmol/h), representing a net release of ammonia to the portal blood relative to time of feeding in lactating Holstein cows. Treatments were 24-h ruminal infusion of water (water INF; circles), 24-h ruminal infusion of 15 g of feed urea/kg of DMI (24-h INF; squares), and 6-h ruminal infusion of 15 g of feed urea/kg of DMI (6-h INF; triangles). Cows were sampled 9 to 15.5 h after termination of 6-h infusion. The net portal flux of ammonia was affected by treatment ($P < 0.01$), reflected by increased net portal flux in 24-h INF compared with water INF and 6-h INF. The ammonia absorption was not different between 6-h INF and water INF during the sampling window. The arrow indicates time of feeding. Data points represent the means of 3 cows $\pm$ standard errors of the means.

dairy cow and it is likely that the capacity of the liver to detoxify ammonia might have been acutely exceeded during the 6-h urea infusion, inducing peripheral hyperammonemia. Steers pulse dosed with 0.5 g of urea/kg of BW (about 16 h after previous feeding) via a ruminal cannula had increased portal and carotid blood ammonia concentrations by 685 and 550%, respectively, 5 min after dosing, and symptoms of toxicity occurred 20 to 30 min after dosing (Bartley et al., 1981). This immediate increase in carotid blood ammonia concentration is an indication that ammonia is able to bypass the liver and enter the systemic circulation (Bartley et al., 1981; Chalmers et al., 1971). According to Bartley et al. (1981), toxic ammonia concentration in arterial plasma is reached at 0.57 mM. The maximal hepatic clearance rate of ammonia in ruminants has been estimated at 70 to 110 mmol/h per kilogram of wet tissue (Symonds et al., 1981; Lobley et al., 1995). Given a liver mass of 10.4 kg (Gibb et al., 1992), the max hepatic uptake rate of ammonia-N can be estimated at 728 to 1,144 mmol/h. In 6-h INF, cows were infused with 968 mmol of urea-N/h and, therefore, were at risk of suffering from ammonia toxicity due to insufficient liver capacity for ammonia detoxification. It can also be speculated that decreased DMI with 6-h INF was related to high ruminal ammonia concentrations during infusion, inducing bacterial lysis and endotoxin release

as known from ruminal acidosis (Nagaraja et al., 1978). We cannot differentiate ruminal effects and systemic ammonia toxicity from ammonia passing the liver during infusion.

The observed effect of the 6-h ruminal urea infusion on DMI in the present study was larger than expected and may have induced systemic changes in N and energy metabolism. This is confirmed by the larger decrease in DMI relative to milk yield with 6-h INF, which resulted in cows being in negative energy balance (29 MJ of NE_L /d). This negative energy balance will have affected tissue mobilization of fat, protein, and minerals to sustain energy demands for milk production, as indicated by increased NEFA concentrations and reduced L-lactate concentrations in arterial blood plasma with 6-h INF compared with other treatments. Also, it is likely that the 6-h INF would have elicited differential responses in hormonal control mechanisms (e.g., catecholamines and growth hormone in response to increased energy demands). Thus, throughout the discussion the effects of treatments are confounded with DMI. Rumen epithelial mass and epithelial blood flows are speculated to be involved in urea-N transfer (Norton et al., 1982; Rémond et al., 1993). Feed intake might affect urea-N transport indirectly via effects on these factors, but no indication exists of specific regula-

Figure 4. Portal-drained viscera (PDV) extraction of arterial urea-N (%) relative to time of feeding in lactating Holstein cows. Treatments were 24-h ruminal infusion of water (water INF; circles), 24-h ruminal infusion of 15 g of feed urea/kg of DMI (24-h INF; squares), and 6-h ruminal infusion of 15 g of feed urea/kg of DMI (6-h INF; triangles). Cows were sampled 9 to 15.5 h after termination of 6-h infusion. A treatment by sampling time interaction ($P = 0.03$) was observed, reflecting a greater PDV extraction ratio of arterial urea-N observed from 0.5 to 3.5 h after feeding in water INF compared with 6- and 24-h INF. From 4.5 to 6.5 h after feeding, the extraction ratios were similar among treatments. The arrow indicates time of feeding. Data points represent the means of 3 cows $\pm$ standard errors of the means.

Table 6. Effect of ruminal urea infusion on renal variables in lactating dairy cows

Item	Treatment ¹				P-value ³		
	Water INF	24-h INF	6-h INF	SEM ²	Trt	Time	Trt × Time
Urine pH	8.0	8.1	7.6	0.18	0.22	0.82	0.19
Plasma creatinine-N, $\mu\text{mol/L}$	241	232	230	2.4	0.11	0.28	0.11
Urine creatinine-N, mmol/L	18 ^a	19 ^a	27 ^b	1.7	<0.01	0.10	0.29
Renal plasma flow, L/h	331 ^a	364 ^b	306 ^a	29.8	0.02	0.64	0.31
Diuresis, L/h	0.93	0.83	0.60	0.11	0.19	0.08	0.46
Creatinine-N clearance, L/h	63	67	63	3.1	0.67	<0.01	0.06
Urea-N clearance, L/h	23 ^a	39 ^b	36 ^b	3.5	<0.01	0.02	0.20
Filtered load of urea-N, mmol/h	184 ^a	639 ^b	495 ^b	52.0	<0.01	0.05	0.04
Urea-N reabsorbed, %	63 ^b	41 ^a	43 ^a	3.7	0.03	0.01	0.08
N constituents in urine, mmol/h							
Total N ⁴	191 ^a	507 ^b	408 ^b	29.5	<0.01	—	—
Urea-N	67 ^a	376 ^b	285 ^b	41.0	<0.01	<0.01	0.07
Hippuric acid-N	23 ^a	29 ^b	22 ^a	1.4	<0.01	0.64	0.11
Creatinine-N	15	15	15	0.6	0.14	<0.01	0.44
Allantoin-N	38 ^{ab}	41 ^b	34 ^a	2.5	0.04	0.03	0.35
Amino acid-N	0.33 ^b	0.44 ^c	0.20 ^a	0.032	<0.01	0.59	0.38
Uric acid-N	4.7	4.5	3.9	0.66	0.35	—	—
N not accounted for	56	50	13	16.3	0.19	—	—

^{a-c}Means within a row with different superscripts differ ($P \leq 0.05$).

¹Treatments were 24-h ruminal infusion of water (water INF), 24-h ruminal infusion of 15 g of feed urea/kg of DMI (24-h INF), and 6-h ruminal infusion of 15 g of feed urea/kg of DMI (6-h INF). Cows were sampled 9 to 15.5 h after termination of 6-h infusion.

² $n = 3$.

³P-values for Trt = treatment, time within sampling day (Time), and Trt × Time interaction effects.

⁴Total N in urine has been corrected for *p*-aminohippuric acid (pAH).

tion of gut urea-N transport based on energy balance of the cow, and the DMI response with 6-h INF should not prevent evaluation of gut urea-N transport. Indeed, the experimental conditions aimed for, that is challenging the urea-N recycling system as to being regulated by either the highest daily ammonia concentration or lowest daily ammonia concentration in the rumen or portal blood, were met successfully.

Ruminal Ammonia Concentration and Ammonia Absorption

Ruminants absorb substantial amounts of ammonia across the PDV. This is apparent from 315 measurements in growing and lactating cattle, where ammonia absorption into the portal blood increased with increasing N intake, with a linear regression slope of 0.42 (Firkins and Reynolds, 2005) which indicates that on an incremental basis, net ammonia absorption into the portal vein accounted for 42% of dietary N intake. Interestingly, in the present study, within the sampling window, the ruminal ammonia concentration as well as the net portal flux of ammonia (Figure 3) with 6-h INF did not differ from water INF. It is speculated that the majority of the ammonia from hydrolyzed urea with the 6-h INF not utilized by rumen microbes was absorbed immediately to the portal blood. This is an important observation with regard to maintaining a

ruminal ammonia availability that sustains ruminal microbial growth. It appears from the present study that short-term supplementation of an easily degradable N source in dairy cows fed a ration deficient in RDP does not leave any ruminal carryover for ammonia to periods when ammonia is in short supply.

Arterial Urea-N Concentration

Infrequent dietary protein supplementation has been investigated as a tool of improving N efficiency in ruminants, with only few negative effects on N balance observed (Reynolds and Kristensen, 2008). Previous work in wethers consuming low-quality forages and supplemented infrequently with protein every 3 or 6 d demonstrated increased plasma urea-N concentrations the day after supplementation (Bohnert et al., 2002). Additionally, Krehbiel et al. (1998) demonstrated in ewes fed low-quality forage and supplemented with soybean meal once every 24 h or every 72 h relative to no supplement, an increased arterial plasma urea-N concentration on the second day following supplementation when ewes were fed every 72 h. In the present study, the observation on arterial blood urea-N concentration with 6-h INF is in line with previous findings of sustained elevated arterial urea-N concentrations after infrequent N supplementation. However, present data also showed that the urea-N removal from the whole

body pool is relatively fast (i.e., the theoretical half life based on liver release and PDV and renal removal of urea-N under the present experimental conditions was approximately 5.5 h). When prolonged elevated urea-N concentrations are observed in studies with infrequent protein supplementation, this effect must partly be due to low degradation rate of the protein source and eventual other protein pools involved (Reynolds and Kristensen, 2008). In the present study, because treatment effects were confounded by the low DMI with 6-h INF, it can be speculated that release of gluconeogenic amino acids from body reserves and urea from infusions might have interacted to increase the blood urea-N concentration by providing N for hepatic synthesis of urea-N via deamination of amino acids and hydrolysis of infused urea-N to ammonia, respectively.

Urea-N Transport

The lack of negative effects with infrequent protein supplementation has been attributed partly to increases in net PDV removal of urea-N during periods of no protein supplementation (Krehbiel et al., 1998; Bohnert et al., 2002; Archibeque et al., 2007). However, these studies in small ruminants and beef cattle examined the effects of rumen asynchrony of N and energy over periods of days. Hence, in the present study, we investigated the effect of infrequent protein supplementation on a daily basis by creating ruminal N and energy asynchrony by short-term urea infusion (6-h INF) on the functional epithelial urea-N transport properties during N shortage relative to a high N supply. The observed lack of increase in absolute urea-N transport across the PDV with 6-h INF within the sampling window despite the sustained elevated arterial blood urea-N concentration contrasts with previous statements implying that urea-N transport across the PDV is responsible for the lack of negative effect on N balance by increasing the ruminal N available for microbial protein synthesis at times when N supply is low (Krehbiel et al., 1998; Cole, 1999; Archibeque et al., 2007). Hence, from the present study, it appears that the cows were unable to make use of circulating urea-N as ruminal N source during periods of the day without intraruminal infusion of urea.

In addition to being recycled across the epithelium of the PDV, urea-N is also transported via saliva to the rumen (Huntington and Archibeque, 2000). However, the contribution via saliva is considered to be of more importance in cattle fed high N levels. This is explained by the high correlation between urea-N concentrations in plasma and saliva (Bailey and Balch, 1961), and further supported by the generally observed downregulation of extraction of arterial urea-N across the epithelia of the rumen at high plasma urea-N concentrations

(Calsamiglia et al., 2010; Røjen et al., 2011b), which reflects a decrease in the ruminal epithelial permeability for urea-N.

In the present study, the treatment by sampling time interaction on PDV extraction of arterial urea-N reflected that the extraction of urea-N in water INF was greater compared with 24-h INF (Figure 4) especially during the first 3.5 h after feeding, and thus supports previous findings of increased urea-N extraction with low arterial urea-N concentrations. However, when evaluating the regulatory effect of daily minimum N status (6-h INF) relative to daily maximum N status (24-h INF), the observed low PDV extraction with 6-h INF during blood sampling was not different from the PDV extraction with 24-h INF despite low ruminal ammonia concentrations. Hence, these data indicate that mechanisms responsible for downregulation of epithelial urea-N transport based on ruminal peak of ammonia or blood urea-N concentrations overruled potential signals from low ruminal ammonia concentration during periods with low ruminal N supply. These observations are also consistent with a previous study indicating that dairy cows were unable to increase urea-N transport to the gut with decreasing RDP (Røjen et al., 2011b). Overall, these observations challenge the hypothesis that urea-N transport functions to mainly supply N to rumen fermentation.

Urea Transporter UT-B

In the kidney, UT proteins help to maintain the osmotic gradient required to concentrate urine (Fenton et al., 2004) but might also be involved in the salvage of urea-N from excretion when animals are fed low-N diets (Isozaki et al., 1994). The detection of UT mRNA in the rumen epithelium of sheep (Ritzhaupt et al., 1998) and cattle (Marini et al., 2004) and the actual characterization of the urea transporter UT-B in the rumen of cattle (Stewart et al., 2005) offer a putative mechanism by which the transport of urea-N across epithelial layers could be explained. However, previous studies in ruminants have not been able to show a clear relationship between UT-B expression and changes in N supply (Marini and Van Amburgh, 2003; Ludden et al., 2009; Røjen et al., 2011a). Additionally, in Ludden et al. (2009), the protein supplementation strategy only had a minor effect on ruminal UT-B expression. In the present study, neither daily maximum concentrations of ammonia in the rumen nor peak in daily blood urea-N concentration lead to any dramatic changes in gut epithelial urea-N transport and neither appeared to have any direct effect on UT-B mRNA expression in ruminal epithelium. Hence, infrequent urea-N supplementation did not lead to changes in UT-B mRNA expression, and

thus is in line with previous studies showing no effect of changes in N supply on UT-B expression. In this study, the confounding effect of DMI and the subsequent negative energy balance could be expected to affect the expression of UT-B mRNA. However, if this were the case, we would expect the UT-B mRNA expression for the 6-h INF to be different from the water INF and 24-h INF. It should be kept in mind that changes in UT-B mRNA expression alone may not be a good indicator of changes in the abundance of the actual UT-B protein. Nonetheless, due to the general lack of correspondence between UT-B mRNA expression and urea-N transport it seems that changes in urea-N transport appears more functional rather than transcriptional and may involve effects of rumen fermentation products on activity of urea transporters other than UT-B that still needs to be identified.

Renal Urea-N Kinetics

When comparing water INF with 24-h INF (low vs. high N supply), the kidneys adapted to the low N supply by decreasing the amounts of total N and urea-N eliminated in the urine, as reported previously in several studies in cattle (Ruiz et al., 2002; Marini and Van Amburgh, 2003; Wickersham et al., 2008). The observed decrease in the renal plasma flow, estimated from the renal clearance of pAH with low N supply is in line with previous reports (Tebot et al., 2002; Røjen et al., 2011b) and is likely to be a contributing factor to the decreased excretion of urea-N for water INF. Additionally, there was no difference between water INF and 24-h INF in creatinine clearance, which is used to estimate glomerular filtration rate. This point toward increased renal reabsorption of filtered urea-N on low N intake (Marini and Van Amburgh, 2003). Indeed, the observed decrease in filtered load of urea-N, along with the increase in urea-N reabsorbed by the kidneys suggests a mechanism salvaging urea-N from excretion when N intake decreases. Contrary to the latter, in 6-h INF where total daily N supply (urea infusion included) was not different from the N supply for water INF treatment, increased renal clearance of urea-N, which did not differ from 24-h INF, as well as increased load of urea-N filtered by the kidney, and decreased percentage of urea-N reabsorbed by the kidney appears to be a consequence of the sustained elevated arterial blood urea-N concentration. These observations show that the priority of the ruminant kidneys is to keep circulating amounts of urea-N within homeostatic range and avoid accumulation of this waste product in the body.

CONCLUSIONS

Short-term ruminal infusion (6 h) of urea in cows fed a basal diet deficient in RDP led to decreased feed intake and prevented treatment comparisons on an isonutritional basis. Short-term urea infusion increased arterial blood urea-N, but not ruminal ammonia concentrations 9 to 15.5 h after termination of infusion. Urea-N transport across the PDV and PDV extraction of arterial urea-N were not increased in the sampling window 9 to 15.5 h after intraruminal urea infusion was terminated. Cows were unable to make use of circulating urea-N as a ruminal N source during the period of the day without ruminal urea infusion. Evaluating the regulatory effect of daily minimum N status relative to daily maximum N status on urea-N transport, implies that ruminal peak concentrations of ammonia or blood urea-N concentrations overruled potential signals from low ruminal ammonia concentration during periods with low ruminal ammonia concentrations.

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