Interaction between dietary content of protein and sodium chloride on milk urea concentration, urinary urea excretion, renal recycling of urea, and urea transfer to the gastrointestinal tract in dairy cows

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ABSTRACT

Dietary protein and salt affect the concentration of milk urea nitrogen (MUN; mg of N/dL) and the relationship between MUN and excretion of urea nitrogen in urine (UUN; g of N/d) of dairy cattle. The aim of the present study was to examine the effects of dietary protein and sodium chloride (NaCl) intake separately, and their interaction, on MUN and UUN, on the relationship between UUN and MUN, on renal recycling of urea, and on urea transfer to the gastrointestinal tract. Twelve second-parity cows (body weight of 645 ± 37 kg, 146 ± 29 d in milk, and a milk production of 34.0 ± 3.28 kg/d), of which 8 were previously fitted with a rumen cannula, were fitted with catheters in the urine bladder and jugular vein. The experiment had a split-plot arrangement with dietary crude protein (CP) content as the main plot factor [116 and 154 g of CP/kg of dry matter (DM)] and dietary NaCl content as the subplot factor (3.1 and 13.5 g of Na/kg of DM). Cows were fed at 95% of the average ad libitum feed intake of cows receiving the low protein diets. Average MUN and UUN were, respectively, 3.90 mg of N/dL and 45 g of N/d higher for the high protein diets compared with the low protein diets. Compared with the low NaCl diets, MUN was, on average, 1.74 mg of N/dL lower for the high NaCl diets, whereas UUN was unaffected. We found no interaction between dietary content of protein and NaCl on performance characteristics or on MUN, UUN, urine production, and renal clearance characteristics. The creatinine clearance rate was not affected by dietary content of protein and NaCl. Urea transfer to the gastrointestinal tract, expressed as a fraction of plasma urea entry rate, was negatively related to dietary protein, whereas it was not affected by dietary NaCl content. We found no interaction between dietary protein and NaCl content on plasma urea entry rate and gastrointestinal urea entry rate or their

ratio. The relationship between MUN and UUN was significantly affected by the class variable dietary NaCl content: $UUN = -17.7 \pm 7.24 + 10.09 \pm 1.016 \times MUN + 2.26 \pm 0.729 \times MUN$ (for high NaCl); $R^2 = 0.85$. Removal of the $MUN \times NaCl$ interaction term lowered the coefficient of determination from 0.85 to 0.77. In conclusion, dietary protein content is positively related to MUN and UUN, whereas dietary NaCl content is negatively correlated to MUN but NaCl content is not related to UUN. We found no interaction between dietary protein and NaCl content on performance, MUN, UUN, or renal urea recycling, nor on plasma urea entry rate and urea transfer to the gastrointestinal tract. For a proper interpretation of the relationship between MUN and UUN, the effect of dietary NaCl should be taken into account, but we found no evidence that the effect of dietary NaCl on MUN is dependent on dietary protein content.

Key words: milk urea nitrogen, urinary urea nitrogen excretion, dietary NaCl, dietary protein

INTRODUCTION

Ammonia emitted from livestock manure has negative environmental effects, including ecosystem acidification, eutrophication of surface waters, and formation of fine particulate matter formation in the atmosphere (Draaijers et al., 1989; Howarth et al., 1996). Moreover, indirect emissions of nitrous oxide (N_2O), which is a major greenhouse gas, occur after atmospheric deposition of ammonia from stables and manure storage (IPCC, 2006). Livestock farming (in particular cattle operations) is considered to be a major contributor to ammonia emission (Pain et al., 1998; Hutchings et al., 2001). The primary source of ammonia emission in dairy production is excreted urinary urea N (UUN; g of N/d). Decreasing the dietary CP content (g/kg of DM) is one of the most effective strategies to decrease total N excretion and ammonia emission from animal manure (Hristov et al., 2011) and may reduce the environmental impact.

Received March 22, 2013.

Accepted June 3, 2013.

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Milk urea N (mg of N/dL) is correlated with UUN, which has led to the development of several predictive models to estimate UUN from MUN (Burgos et al., 2007; Powell et al., 2011). These models assume a fixed increase of UUN per unit increase of MUN. However, several factors can affect the relationship between MUN and UUN, including BW and cow genetics, time of sampling, protein content of the diet, and the quantity of urine produced (Kauffman and St-Pierre, 2001; Aguilar et al., 2012; Spek et al., 2013). In a study by Spek et al. (2012), MUN was negatively related to NaCl intake, whereas UUN was not affected. From a physiological perspective, renal regulation of urea excretion can explain the effect of dietary CP and urine volume on the relationship between MUN and UUN. Several studies in goat, sheep, and steers show that renal processes such as the glomerular filtration rate (GFR) and recycling of urea from the glomerular filtrate are affected by CP (Thornton, 1970; Rabinowitz et al., 1973; Eriksson and Valtonen, 1982) and NaCl (Thornton, 1970; Godwin and Williams, 1984). Because both NaCl and protein intake may affect GFR and urea recycling, they might have an interactive effect on the process of renal urea excretion and MUN. The interaction between NaCl and protein on plasma urea nitrogen concentration (PUN; mg of N/dL) and on renal handling of urea was studied by Thornton (1970) in 2-yr-old steers. Addition of NaCl (200 g of NaCl/d) reduced PUN by 42% from 3.48 to 2.02 mg of N/dL at the high CP level (66.9 g of CP/kg of DM), whereas it reduced PUN by only 19% from 1.15 to 0.93 mg of N/dL at the low CP level (41.6 g of CP/kg of DM). Urinary urea N excretion more than doubled upon NaCl addition at the low CP level, but NaCl addition did not affect UUN at the high CP level. Such differences suggest an interactive effect of protein and NaCl intake on the change in UUN per unit of change in PUN. Because MUN is a good indicator of PUN (Roseler et al., 1993), a similar interaction can be expected for MUN. It is unclear, however, whether such an interaction can be expected in lactating dairy cattle because dietary concentrations of CP in dairy cattle rations in practice are 3 to 4 times higher than the 40 to 70 g of CP/kg of DM investigated by Thornton (1970). Furthermore, DMI in dairy cattle is substantially higher than that in steers in the study of Thornton (1970).

The aim of the present study was to examine the effects of protein and NaCl intake separately, and their interaction, on MUN, PUN, UUN, the relationship between MUN and UUN, and the GFR and reabsorption of urea from the glomerular filtrate. Another aim was to study the effect of dietary CP and NaCl on urea transfer to the gastrointestinal tract.

MATERIALS AND METHODS

Cows, Housing, and Experimental Design

The experiment was approved by the Institutional Animal Care and Use Committee of the Animal Sciences Group, Wageningen University and Research Centre (Lelystad, the Netherlands). Twelve second-parity cows, of which 8 had a rumen fistula, were selected based on milk production and presence of a rumen fistula. At the start of the experiment, the cows had BW of 645 ± 37 kg, milk production of 34.0 ± 3.28 kg/d, and were 146 ± 29 d in milk (means $\pm$ SD). Cows were housed in a tiestall to quantitatively collect urine and feces. Cows were blocked into 3 groups, according to milk production and presence of a rumen fistula. Cows within blocks were randomly assigned to 1 of 4 treatments. Treatments consisted of 2 dietary concentrations of protein (116 and 154 g of CP/kg of DM) and for each protein content 2 concentrations of NaCl (3.1 and 13.5 g of Na/kg of DM). The ingredients and chemical composition of the diets is presented in Table 1. The experiment had a split-plot arrangement with cow and dietary CP content as the main plot factors and NaCl content as the subplot factor. Each cow received in total 2 dietary treatments. Cows were assigned to either a low or a high protein diet, and within protein level received a low and a high dietary NaCl content, resulting in $n = 24$ observations. Because of practical limitations, measurements could be carried out on only 6 animals per day. Therefore, the 12 cows were divided into 2 groups of 6 cows each; 7 d after the first group of 6 cows entered the experiment, the second group of 6 cows entered the experiment. This resulted in a partial balanced design with 4 treatments and 6 cows per measurement period and a total number of 4 measurement periods. For each cow, the total length of the study was 38 d. The first 13 d consisted of an adaptation period to the diet with ad libitum feed access; from d 14 until the end of the experiment, cows were fed at 95% of the average ad libitum feed intake based on the cows receiving the low protein diets, excluding NaCl addition. The first 2-d measurement period (collection d 24 and 25) was followed by a 10-d adaptation period to the new diet (high or low NaCl) and followed by a second 2-d measurement period (collection d 37 and 38).

Cows were milked twice daily at 0500 and 1700 h throughout the experiment. During the noncollection days, cows were fed 2 equal meals twice daily at 0500 and 1700 h, whereas during collection days, 67% of the daily feed allowance was provided in 8 equal meals every 2 h, starting at 0500 h until 1900 h, to minimize variation in PUN and MUN caused by variation in feed

Table 1. Dietary composition (g/kg of DM unless otherwise stated) of experimental diets

Composition	Low protein		High protein	
	Low NaCl	High NaCl	Low NaCl	High NaCl
Ingredient				
Corn silage ¹	656	640	656	640
Wheat straw, chopped	44	43	44	43
Soybean hulls	205	199	106	103
Soybean meal protected ²			131	127
Soybean meal (53% CP in DM)	47	46	27	27
Limestone	13	13	13	13
Vinasses	5.8	5.7	5.9	5.7
Palm fatty acids	8.3	8.1	1.3	1.3
Urea	5.5	5.4	2.8	2.8
Feed salt ³	5.3	29.9	5.0	29.6
Monocalcium phosphate	5.6	5.5	2.6	2.6
Kieserite	1.6	1.6	1.7	1.6
Magnesium oxide	0.8	0.8	0.7	0.6
Mineral and vitamin premix ⁴	2.3	2.2	2.3	2.3
Nutrient				
DM (g/kg of feed)	473	481	473	473
CP	119	114	156	151
Ash	70.3	95.1	71.0	95.7
Crude fat	33.5	34.2	29.8	31.9
Starch	239	225	239	226
NDF	383	412	362	372
ADF	289	285	261	256
Ca	8.5	8.1	7.6	7.4
K	10.4	10.2	11.5	10.9
Na	3.0	14.6	3.2	12.3
Feeding value parameter				
DVE ⁵	69	67	105	102
OEB ⁶	−9	−9	−9	−9
NE _L ⁷ (MJ/kg of DM)	6.61	6.45	6.63	6.47
Rumen-degradable CP	80	78	80	78

¹Corn silage (g/kg of DM unless specified otherwise): DM, 357 g/kg; CP, 73; starch, 374; NDF, 320; ADF, 180; ADL, 14; ash, 47 (determined with near-infrared spectrometry; BLGG, Wageningen, the Netherlands).

²Formaldehyde-treated soybean meal (540 g of CP/kg of DM).

³Composition of feed salt: ≥99.8% NaCl.

⁴Contained per kilogram of mix: 48 g of Ca; 0.8 g of Mg; 6,263 mg of Cu; 5,010 mg of Mn; 10,048 mg of Zn; 501 mg of I; 150 mg of Co; 160 mg of Se; 2,505,010 IU of vitamin A; 601,202 IU of vitamin D₃; and 6,263 IU of vitamin E.

⁵Intestinal digestible protein (Van Duinkerken et al., 2011).

⁶Rumen-degraded protein balance (Van Duinkerken et al., 2011).

⁷Calculated with VEM (feed unit lactation) system (Van Es, 1975).

intake within a day. At 2100 h, the remaining 33% of the total daily feed allowance was provided. Daily individual feed intake was determined by subtracting the quantity of orts from the quantity of feed supplied.

Sample Collection

The diets were prepared daily by using a paddle mixer (Holaras V.DC 1200, Hoopman Machines BV, Aalten, the Netherlands), and a representative sample (~700 g) of each TMR was collected daily and stored at −20°C. Samples of each diet for each treatment week were pooled and stored at −20°C pending analyses. During sample collection days, milk yield was determined after

each milking, and milk samples (10 mL) were obtained after each milking from the total quantity of milk. Milk samples were stored at −20°C in tubes containing sodium azide pending analyses.

One day before each 2-d sample collection period, cows used for sample collection were fitted with indwelling Foley urine catheters (Ch. 26, 30-mL balloon; Bard Ltd., Crawley, UK) attached to a collection vessel. Urine catheters were removed from the cows directly after each sample collection period. The animals were also fitted with 2-way blood-sampling catheters (BD Careflow dubbel lumen, Becton Dickinson BV, Breda, the Netherlands) in the jugular vein 2 d before cows were used for the first time of sample collection.

Catheters were kept open by flushing with saline solution containing 33 U of heparin/mL throughout the experiment. At each sample collection day at 0902 h, an amount of 0.98 ± 0.020 and 1.92 ± 0.016 g of [^{13}C] urea (99% ^{13}C enriched, Sigma-Aldrich, Zwijndrecht, the Netherlands) dissolved in 10 mL of saline was injected intravenously in cows on the low and high protein diets, respectively. Following infusion, the syringe was emptied and subsequently filled and emptied with blood twice to ensure that the whole [^{13}C]urea dose entered the bloodstream. Blood samples were collected at 0900, 0922, 1000, 1100, 1200, 1300, 1500, 1700, and 2100 h. These blood samples (10 mL) were collected in heparin tubes and centrifuged within 2 h ($3,000 \times g$ for 15 min at 4°C); then, the blood plasma was separated and collected before being stored at -20°C . Urine and feces were collected quantitatively for 24 h during each of the 2 consecutive collection days starting at 0500 h. Production of urine and feces was recorded after each collection day; urine and feces were weighed, thoroughly mixed, sampled (~ 250 mL of urine, ~ 200 g of feces), and stored at -20°C . After the first measurement day, the urine collection vessel was replaced by a new vessel containing 364 g of 48% H_2SO_4 . Before a urine sample was taken, the pH of the collected urine was measured and additional H_2SO_4 was added if the pH value was above 3.0. The weight of urine recorded and urine analyses were corrected for weight and volume of sulfuric acid added. During sample collection days, at 0445 h and 1645 h, rumen liquid samples (30 mL) were obtained from 4 cows with a rumen fistula, put directly in ice water, and centrifuged within 30 min ($3,000 \times g$ for 15 min at 4°C); the supernatant was stored at -20°C .

Analytical Procedures

Dry matter content of TMR samples was determined after oven drying at 70°C during 24 h after which the dried samples were ground in a cross beater mill (Peppink, Deventer, the Netherlands) to pass a 1-mm screen. Samples were analyzed for DM (EC 152/2009; European Union, 2009), ash (EC 152/2009; European Union, 2009), N (ISO 5983; ISO, 1997), crude fat (EC 152/2009; European Union, 2009), NDF (ISO 16472; ISO, 2006), ADF (ISO 13906; ISO, 2008), starch (ISO 6493; ISO, 2000a), and the minerals Ca, Na, and K (ISO 6869; ISO, 2000b) by Pre-Mervo (Utrecht, the Netherlands). Milk was analyzed for fat, protein, lactose, and SCC as described by Abrahamse et al. (2008). Milk urea content was determined using the pH-difference technique (ISO 14637; ISO, 2004). Urea concentration in acidified urine and blood plasma samples collected at 0800, 1200, and 1700 h was analyzed using the urea

liquicolor test (Human, Wiesbaden, Germany), which is based on measuring absorbance of light (578 nm) after a modified Berthelot reaction. Feces and acidified urine were analyzed for N by the Kjeldahl method with CuSO_4 as the catalyst (ISO 5983; ISO, 1997). Urine was mixed and, during mixing, a sample was taken for analysis of N to include a proportional part of the precipitate as well. Feces were analyzed for DM (ISO 6496; ISO, 1999). Blood plasma and acidified urine samples were analyzed for creatinine by the Veterinary Diagnostic Laboratoria of Utrecht University (Utrecht, the Netherlands) by measuring absorbance of light (520 nm) after a modified Jaffé reaction. Creatinine was analyzed in the individual blood plasma samples collected at 0800, 1200, and 1700 h, to obtain an estimate of the average daily plasma creatinine concentration. Because creatinine was analyzed on a volume basis, it was necessary to estimate the volume of urine produced (as the weight and volume of added sulfuric acid is known). To this end, the specific gravity of the urine produced is required. Dividing the weight of urine by the specific gravity of urine gives the volume of urine produced. The specific gravity of urine in this study was estimated based on the relationship between specific gravity of urine and the daily urine production (kg/cow per day) determined in a previous study of Spek et al. (2012; data on specific gravity unpublished; Figure 1). Ammonia (NH_3) in rumen liquid was determined colorimetrically using a spectrophotometer (Cary 50, Varian, Palo Alto, CA) based on the Berthelot reaction as described by Searle (1984) after deproteinizing the supernatant by addition of 10% TCA. Enrichment of [^{13}C]urea in blood plasma was analyzed by gas chromatography-combustion-isotope ratio mass spectrometry (GC-C-IRMS; GC type Finnigan Trace GC Ultra, Thermo Electron Corporation, Milan, Italy; IRMS type Delta V Advantage, Thermo Scientific, Bremen, Germany), using the procedure described by Dai et al. (2010).

Calculations

The creatinine (Crea) clearance rate (**CCR**, L/min), representing the GFR or the volume of blood being filtered by the kidneys was calculated as

$$\frac{\text{Total daily urinary Crea excretion (mmol/d)}}{\text{Blood plasma Crea concentration (mmol/L)}} \div 1,440 \text{ (min/d)}.$$

The urea clearance rate (**UCR**, L/min) was calculated as

$$\frac{\text{Total daily urinary urea excretion (mg/d)}}{\text{Blood plasma urea concentration (mg/L)}} \div 1,440 \text{ (min/d)}.$$

Figure 1. Relationship between urine specific density (g/mL) and urine production (kg/cow per day, where variation in urine was caused by differences in NaCl intake [unpublished data of the specific gravity in urine in the study by Spek et al. (2012)]). Urine specific density (g/mL) = $1.0356 \pm 0.00091 + 0.134 \pm 0.0590 \times \exp(-0.121 \pm 0.0267 \times \text{urine production (kg/cow per d)})$; $R^2 = 0.74$.

The renal urea reabsorption ratio (**RRR**) was calculated as: $1 - (\text{UCR}/\text{CCR})$. The N-balance was calculated as N-intake minus N excreted in milk, feces, and urine. Urea-N transfer to the gastrointestinal tract (**GER**) was calculated as the plasma urea-N entry rate (**UER**; g urea N/d) minus UUN and milk urea-N excretion rate (**MUE**; g of urea-N/d):

$$\begin{aligned} \text{GER (g of urea-N/d)} &= \text{UER (g of urea-N/d)} \\ &- \text{UUN (g of urea-N/d)} - \text{MUE (g of urea-N/d)}; [1] \end{aligned}$$

UER was calculated by multiplying the fractional disappearance rate of plasma urea (K_{urea} ; /h) under steady-state condition with the plasma urea pool (**PUP**; g of urea-N) and then multiplied by 24:

$$\begin{aligned} \text{UER (g of urea-N/d)} &= K_{\text{urea}} (/h) \\ &\times \text{PUP (g of urea-N)} \times 24. [2] \end{aligned}$$

Correction of the $[^{13}\text{C}]$ urea atom percentage for natural enrichment (measured immediately before infusion at 09:00 h) resulted in atom percent excess (**APE**; %) values for $[^{13}\text{C}]$ urea. The APE specifies the level of isotopic abundance above a given background level. The background level is subtracted from the experimental value to give the APE value. The K_{urea} was calculated by fitting an exponential model to the APE $[^{13}\text{C}]$ urea enrichment data of plasma from 1000 until 2100 h:

$$\text{APE } [^{13}\text{C}] \text{urea (\%)} = A \times \exp(-K_{\text{urea}} \times \text{time}), [3]$$

where A is the estimated APE of $[^{13}\text{C}]$ urea % at 0902 h (the time of infusion of $[^{13}\text{C}]$ urea).

The PUP was calculated by dividing the quantity of injected $[^{13}\text{C}]$ urea by the APE $[^{13}\text{C}]$ urea % fitted at 0902 h using equation [3]:

$$\text{PUP (g of urea-N)} = \text{g of } [^{13}\text{C}] \text{urea} / (A/100) \times 28/60. [4]$$

Statistical Analysis

Observations per cow per sample collection period were averaged before statistical analysis. Effect of dietary protein and NaCl as class variables and the interaction between dietary protein and NaCl on dependent variables were evaluated as a split plot with protein content as main plot and NaCl content and interaction of protein with NaCl as subplot using the MIXED procedure in SAS (version 9.2, SAS Institute Inc., Cary, NC) with dietary protein, NaCl, and protein $\times$ NaCl interaction included as fixed effects, and cow nested within protein content as random effect. The dependent variables MUN, PUN, urinary N (**UN**; g of N/d), and UUN were log-transformed before analyzing significance levels of NaCl, protein, and the interaction between NaCl and protein, as the residuals of these variables tended to inflate as these values increased. Standard errors reported in the tables are the standard errors of the least squares means.

Calculation of error degrees-of-freedom was done by the DDFM = KENWARDROGER method in PROC MIXED and the covariance structure was modeled as compound symmetry. Estimation of A and K_{urea} was carried out by using the NLIN procedure of SAS. The effect of dietary NaCl as a class variable on the relationship between UUN and MUN (neither UUN nor MUN was log-transformed) was tested with the GLM procedure of SAS with UUN as the dependent variable and MUN and the MUN $\times$ dietary NaCl interaction as independent variables. Significance was declared at $P < 0.05$, and tendencies at $0.05 \leq P < 0.10$.

RESULTS

Cows, Diets, Milk Production, and MUN

One case of mastitis and 1 case of a blocked urine catheter occurred during the measurement days, resulting in 2 missing values in the data set for $n = 22$ observations. The average difference in Na-intake between the high and low NaCl diets was 199 g of Na/d (Table 2), and the average difference in N-intake between the high and low protein diets was 130 g of N/d (Table 3). The DMI tended to be higher ($P = 0.065$) for the cows receiving the high protein diets compared with cows receiving the low protein diets (Table 2). Com-

Table 2. Effect of dietary protein and NaCl content on intake of Na and N, DMI, milk production, milk composition, rumen ammonia concentration, and apparent digestibility of feed DM in lactating dairy cows¹

Parameter	Low protein		High protein		SE	<i>P</i> -value		
	Low NaCl	High NaCl	Low NaCl	High NaCl		Protein	NaCl	Protein × NaCl
Na-intake (g/d)	58	273	62	244	10.2	0.263	<0.001	0.110
DMI (kg/d)	19.2	19.3	19.6	20.0	0.28	0.068	0.383	0.507
Milk yield (kg/d)	22.4	22.0	24.7	26.5	1.03	0.028	0.285	0.126
FPCM ² (kg/d)	23.3	23.7	25.0	26.1	1.18	0.228	0.115	0.517
MUN (mg of N/dL)	5.29	3.66	9.29	7.45	0.342	<0.001	0.002	0.314
Fat (%)	4.35	4.62	4.08	3.97	0.375	0.385	0.644	0.265
Protein (%)	3.34	3.45	3.48	3.29	0.142	0.961	0.691	0.162
Lactose (%)	4.48	4.54	4.59	4.54	0.099	0.651	0.907	0.379
SCC (× 1,000 cells/mL)	144	185	90	100	33.3	0.123	0.279	0.500
Rumen NH ₃ (mg of N/L)	32.9	36.7	45.4	36.2	4.25	0.257	0.554	0.182
ATTD ³ (%)	65.4	67.2	70.2	70.4	0.92	0.002	0.265	0.390

¹Values are least squares means.²Fat- and protein-corrected milk: $(0.337 + 0.116 \times \text{fat \%} + 0.06 \times \text{protein \%}) \times \text{milk yield (kg/d)}$.³Apparent total-tract digestibility of DM.

pared with that in the low protein treatments, milk production in the high protein treatments was 3.4 kg/d higher ($P = 0.028$; Table 2). However, milk production corrected for fat and protein content (FPCM) did not differ significantly between low and high protein treatments. Milk concentrations of fat, protein, lactose, and SCC were not related to dietary content of protein and NaCl. Milk urea nitrogen concentration was positively affected by protein intake ($P < 0.001$) and negatively by NaCl intake ($P = 0.002$). Compared with that of the low protein treatments, MUN was 3.90 mg of N/dL higher for the high protein treatment. Compared with that of the low NaCl treatments, MUN was 1.74 mg of N/dL lower for the high NaCl treatments. We observed no interaction effect between dietary content of protein and NaCl on MUN ($P = 0.314$) or on milk production and milk composition (Table 2).

UUN-MUN Relationship

We found a significant interaction effect ($P = 0.006$) of dietary NaCl (class variable) on the relationship between UUN and MUN: $\text{UUN (g of N/d)} = -17.7 \pm 7.24 + 10.09 \pm 1.016 \times \text{MUN (mg of N/dL)} + 2.26 \pm 0.729 \times \text{MUN (mg of N/dL)} \times \text{NaCl (for high NaCl)}$, $R^2 = 0.85$ (Figure 2). Removal of the MUN × NaCl interaction term lowered the coefficient of determination from 0.85 to 0.77.

Nitrogen Flows

Milk-N, fecal-N, and UN were all affected by protein content but not by NaCl content (Table 3). Compared with the low protein diets, high protein diets increased excretion of milk-N (19 g of N/d), fecal-N (16 g of

Table 3. Effect of dietary protein and NaCl content on excretion of N in milk, urine, and feces, and on urea and non-urea N excretion in lactating dairy cows¹

Parameter (g of N/d)	Low protein		High protein		SE	<i>P</i> -value		
	Low NaCl	High NaCl	Low NaCl	High NaCl		Protein	NaCl	Protein × NaCl
N-intake	361	350	488	482	11.8	<0.001	0.492	0.820
N-milk	120	122	138	141	5.0	0.021	0.371	0.912
N-feces	137	128	149	148	4.4	0.008	0.228	0.369
N-urine	77	69	128	130	7.2	<0.001	0.495	0.373
N-balance ²	24	31	67	64	11.2	0.023	0.784	0.562
UUN ³	34	28	77	74	5.6	<0.001	0.269	0.445
NUUN ⁴	43	41	51	55	3.9	0.013	0.725	0.436

¹Values are least squares means.²Calculated as N-intake minus excretion of N in milk, feces, and urine.³Urinary urea-N excretion.⁴Urinary non-urea-N excretion.

Figure 2. Relationship between MUN concentration (mg of N/dL) and urinary urea nitrogen excretion (UUN; g of N/d) for low NaCl (3.1 g of Na/kg of DM; dashed regression line) and high NaCl (12.9 g of Na/kg of DM; solid regression line) diets. $UUN = -17.7 \pm 7.24 + 10.09 \pm 1.016 \times MUN + 2.26 \pm 0.729 \times MUN$ (for high NaCl); $R^2 = 0.85$.

N/d), UN (56 g of N/d), UUN (46 g of N/d), and non-urinary-urea-N (11 g of N/d). Also, the average N-balance was 38 g of N/d higher for the high protein diets. No interaction between dietary content of protein and NaCl on N-flows was present (Table 3).

Renal Functioning

Compared with the low NaCl diets, the high NaCl diets had, on average, a 116% higher urine production (32.3 vs. 15.0 kg/d), a tendency ($P = 0.080$) toward a lower plasma creatinine concentration (63.7 vs. 66.4 $\mu\text{mol/L}$), a 16% lower PUN (9.10 vs. 10.86 mg of N/dL), and a 20% higher UUN:MUN ratio (8.91 vs. 7.40) (Table 4). Compared with the low protein diets, the high protein diets had a 68% higher PUN (12.51 vs. 7.45 mg of N/dL), a 29% higher UUN:MUN ratio (9.17

vs. 7.13), and a 45% increase in renal UCR (L/min; 0.421 vs. 0.290 L/min). We observed no interaction between dietary content of NaCl and protein on any parameter tested (Table 4).

Rumen Ammonia and Feed Digestibility

Apparent total-tract digestibility of feed dry matter (ATTD; %) was positively related to dietary protein content but not to dietary NaCl content (Table 2). The concentration of rumen NH_3 was not related to dietary protein (Table 2).

Urea Transfer to the Gastrointestinal Tract

The [^{13}C]urea observations at 0922 h were not included in the analysis as these observations were too high, highly variable, and not in line with the decline in [^{13}C]urea observed for the other sampling time points (Figure 3), resulting in a substantially reduced model fit. Presumably, the injected [^{13}C]urea bolus did not completely disperse over the entire urea distribution volume within the first 20 min after infusion. The fractional disappearance rate of plasma urea was negatively related to dietary protein content, whereas PUP, UER, and GER were positively related (Table 5). As a fraction of dietary N-intake, UER and GER were not significantly affected by dietary protein level or by dietary NaCl level. However, the GER:UER ratio, or the fraction of UER that is returned to the gastrointestinal tract, was negatively related to dietary protein level (Table 5). The UER was negatively related to dietary NaCl level, whereas K_{urea} , PUP, the GER:N-intake ratio, and the GER:UER ratio were not related to dietary NaCl level. The GER ($P = 0.076$) and the UER:N-intake ratio ($P = 0.076$) tended to be negatively related to dietary NaCl.

Table 4. Effect of protein and NaCl content on urine volume, urinary creatinine excretion (CREA), plasma creatinine concentration (PCC), plasma urea nitrogen concentration (PUN), ratio between urinary urea nitrogen excreted (UUN; g of N/d) and the concentration of MUN (mg of N/dL), urea clearance rate (UCR), creatinine clearance rate (CCR), and renal recycling ratio of urea (RRR) in lactating dairy cows¹

Parameter	Low protein		High protein		SE	P-value		
	Low NaCl	High NaCl	Low NaCl	High NaCl		Protein	NaCl	Protein $\times$ NaCl
Urine ² (kg/d)	13.1	31.4	17.0	33.1	1.51	0.159	<0.001	0.356
CREA (mmol/d)	99	98	103	109	6.4	0.271	0.685	0.585
PCC ($\mu\text{mol/L}$)	68.4	65.3	64.3	62.1	2.34	0.252	0.080	0.745
PUN (mg of N/dL)	8.36	6.53	13.35	11.66	0.398	<0.001	<0.001	0.219
UUN:MUN	6.50	7.76	8.29	10.05	0.652	0.006	0.032	0.708
UCR (L/min)	0.282	0.297	0.400	0.441	0.0264	<0.001	0.308	0.621
CCR (L/min)	1.015	1.070	1.128	1.221	0.0870	0.168	0.410	0.824
RRR	0.698	0.697	0.640	0.639	0.0277	0.141	0.934	0.991

¹Values are least squares means.

²Excluding sulfuric acid added to the urine collection vessels.

Table 5. Effect of dietary protein and NaCl on plasma urea pool size and urea transfer characteristics in lactating dairy cows¹

Parameter	Low protein		High protein		SE	P-value		
	Low NaCl	High NaCl	Low NaCl	High NaCl		Protein	NaCl	Protein × NaCl
K _{urea} ² (/h)	0.313	0.339	0.268	0.271	0.0186	0.014	0.447	0.527
PUP ³ (g of urea-N)	30.6	25.5	48.9	45.6	2.48	<0.001	0.108	0.726
UER ⁴ (g of urea-N/d)	226	199	315	292	11.8	<0.001	0.020	0.825
GER ⁵ (g of urea-N/d)	189	170	234	215	12.4	0.012	0.076	0.960
UER:N-intake	0.630	0.568	0.652	0.607	0.0330	0.455	0.076	0.760
GER:N-intake	0.531	0.486	0.485	0.448	0.0346	0.297	0.213	0.907
GER:UER	0.842	0.853	0.744	0.737	0.0211	<0.001	0.904	0.678

¹Values are least squares means.²Fractional disappearance rate of plasma urea-N.³Plasma urea pool.⁴Plasma urea entry rate.⁵Gastrointestinal urea entry rate.

DISCUSSION

Cows, Diets, Milk Production, and MUN

Although this study aimed to minimize differences in DMI between treatments by feeding cows at 95% of the average ad libitum feed intake for the low protein diets during the adaptation period, DMI for the high protein treatments tended ($P = 0.068$) to be higher by 0.6 kg/d. A 6% higher ATTD was observed for the high protein diets. This finding indicates reduced fermentation of feed in the gastrointestinal tract at the low protein level, which may have caused an increased rumen fill and reduced feed intake. A suboptimal rumen NH_3 concentration would be a logical explanation for the reduced ATTD at the low protein diets. However, level of rumen NH_3 was not significantly affected by dietary

protein content (Table 2), and low and high protein diets were formulated in such a way to have similar quantities of RDP and a similar RDP balance (Table 1). The slightly negative dietary RDP balance resulted in NH_3 levels (32.9–45.4 mg of N/L) that were below the levels of NH_3 considered sufficient for optimal microbial protein synthesis (Satter and Slyter, 1974). Milk production was higher for the high protein diets (25.6 kg of milk/d) compared with the low protein diets (22.2 kg of milk/d). This difference of 3.4 kg of milk/d is slightly lower than the milk yield response to changes in dietary CP content predicted according to NRC (2001; namely, 4.1 kg/d). However, differences in FPCM were much smaller and not significantly different. Compared with the low protein diets, the 15% higher, on average, N excretion in milk for the high protein diets suggests that the low protein diet limited milk production. Several factors might explain this lower milk production for the low protein diets in the present study. First, the ATTD of feed was reduced for the low protein diets, resulting in less energy available for milk protein production and possibly less efficient utilization of absorbed protein for milk protein synthesis. Second, the feed intake for the low protein diets was some 0.6 kg of DM/d lower, resulting in less energy and protein available for milk protein production. Third, the ratio of urea-N to total dietary N in the low protein diets (0.136) was higher than that in the high protein diets (0.053) in the present study. Several studies have shown that the ratio of true protein to dietary CP is positively related to milk production (Polan et al., 1976; Brito and Broderick, 2007; Broderick and Reynal, 2009). The effect of dietary protein concentration on MUN in this study is in line with results from other studies with similar ranges in dietary CP content (Korhonen et al., 2002; Colmenero and Broderick, 2006). The average decrease in MUN of 0.88 mg of N/dL per 100 g/d of Na intake

Figure 3. Example of an average result of a regression (excluding the data point at 20 min after injection of a ^{13}C urea bolus; see Materials and Methods for further explanation) of dilution of ^{13}C urea in plasma after injection of a ^{13}C urea bolus. APE = atom percent excess.

is similar to the decrease in observed PUN in the study of Weeth and Haverland (1961) of 0.85 mg of N/dL per 100 g/d of Na intake, but larger than the decrease in MUN of 0.68 mg of N/dL observed in another recent trial by Spek et al. (2012). No interaction between dietary NaCl and protein content on MUN was found. An interaction effect was anticipated based on the 42 and 19% decreases of PUN upon supply of 200 g of Na/d observed with high and low protein diets, respectively, in steers by Thornton (1970). Dietary concentrations of protein in the study of Thornton (1970) of 67 and 42 g of CP/kg of DM for the high and low protein diets, respectively, were substantially lower than the 154 and 116 g of CP/kg of DM in the present study. Perhaps an interactive effect of dietary NaCl and protein on MUN or PUN is only present at extremely low dietary protein contents.

UUN-MUN Relationship

Compared with the low salt diets, the higher ($P = 0.006$) excretion of 2.26 ± 0.729 g of urinary urea-N per unit increase in MUN for the high salt diets was in line with results from Spek et al. (2012), where dietary NaCl was negatively related to MUN but not to UUN. The amount of explained variation in UUN increased from 77 to 85% when NaCl level was included in the model. The variation in dietary urine levels of 13.1 to 33.1 kg/d in the present study, caused by variation in NaCl intake, is observed in practice and comparable to urine production levels observed by De Campeneere et al. (2006) for diets based on maize silage (14.4 L/d) and grass silage (35.0 L/d). This improved explanation of UUN by taking NaCl level, as well as MUN, into account is relevant for the dairy industry because MUN is often used as an indicator for protein nutrition and UUN.

Nitrogen Flows

The positive effect of dietary CP content on N-excretion in milk is consistent with other studies that varied concentrations of CP (Gonda et al., 1995; Korhonen et al., 2002; Colmenero and Broderick, 2006). Colmenero and Broderick (2006) observed a reduction in absolute fecal N-excretion upon an increase in dietary CP from 13.5 to 15.0% in DM, and Ruiz et al. (2002) observed no absolute increase in fecal N excretion upon an increase in CP from 11.1 to 14.1% in DM. Both studies show that excretion of fecal N did not increase as levels of dietary CP increased. This was, however, not the case in the present study, probably as the result of (1) the high content of formaldehyde-treated soybean meal in the high protein diets that was less digestible than

the high content of urea and untreated soybean meal provided in the low protein diet, and (2) the average 0.6-kg-higher DMI for the high protein diets as DMI is positively related to fecal N excretion (Huhtanen et al., 2008). Also, the quantity of non-urea-urinary-N (NUUN; g N/d) was larger for the high protein diets. This result might be attributed to a higher excretion of purine derivatives in the urine originating from rumen-synthesized microbial protein. More microbial protein may have been synthesized with the high protein diets because of the higher efficiency of microbial protein synthesis on true protein compared with ammonia as the N source in RDP (Argyle and Baldwin, 1989; Chikunya et al., 1996; Dijkstra et al., 1998). Such variation in type of urinary N excreted is of relevance in view of environmental issues, because various urinary N constituents differ widely in their effect of N_2O release (Dijkstra et al., 2013).

A positive N balance was established for the low protein diets (on average 28 g of N/d, representing 8% of daily N intake) and an even larger N-balance for the high protein diets (on average 66 g of N/d, representing 14% of daily N intake). This positive N-balance could not be ascribed to increased N retention by the animals as the average BW for the low and high protein treatments at the end of the trial was 19.8 ± 16.30 and 10.6 ± 12.17 kg lower, respectively, than at the start of the trial. The positive N-balance in the present study is in line with the average N-balance (39 g of N/d) reported by Spanghero and Kowalski (1997) in a review on dairy cattle N-balance trials. Spanghero and Kowalski (1997) concluded that volatile N losses from feces and urine, errors in milk N analysis, and dermal N and scurf N losses together cannot account for the average positive N balance observed in their study. Other possible sources of N losses that contribute to a positive N-balance are gaseous N (Costa et al., 1968) and excretion of N in urine as nitrate, which is not detected by the Kjeldahl method (Young et al., 1981). In line with the results in our study, Spanghero and Kowalski (1997) observed a positive relationship between N-balance and dietary N availability. However, we have no conclusive evidence on the background of the increase in positive N-balance observed with the high protein treatment in the present study.

Renal Functioning

The magnitude of the positive effect of NaCl intake on urine production was around 0.082 kg of urine per g of Na intake in this study, which is somewhat smaller than the slope of 0.115 established in a meta-analysis of Bannink et al. (1999) and 0.136 observed in an earlier trial (Spek et al., 2012) for lactating cows, but larger

than the slope of 0.037 found in steers weighing 325 kg (Thornton, 1970). The size of the effect of NaCl intake on urine volume may also be affected by other factors related to cow, diet, and environment. The negative relationship between dietary NaCl and PUN ($P < 0.001$) and between dietary NaCl and plasma creatinine concentration ($P = 0.080$) can be explained by an increase in the GFR, as the total daily excretion of urea and creatinine in urine was not affected by dietary NaCl. However, although CCR was numerically 7% higher for the high NaCl diets compared with the low NaCl diets, no significant effects of dietary NaCl on CCR ($P = 0.410$) were established. Previously, Spek et al. (2012) also did not observe an effect of NaCl intake on CCR. In the study by Spek et al. (2012), a positive linear plateau relationship was observed between RRR and CCR. That relationship implies that with an increase of urea transport to the kidneys more urea is recycled to blood. This makes sense as a mechanism for the cow to maintain a certain urea plasma concentration required for urea recycling to the gastrointestinal tract. However, in the present study, a positive relationship between RRR and CCR was found only for the low protein observations ($P = 0.023$), whereas no significant relationship between RRR and CCR for the high protein observations was observed ($P = 0.482$). The UUN was positively related to CCR ($P = 0.002$, $R^2 = 0.66$) and tended to be negatively related to RRR ($P = 0.058$, $R^2 = 0.34$) for the high protein diets, whereas no significant effect of CCR ($P = 0.983$, $R^2 = 0.00$) and RRR ($P = 0.156$, $R^2 = 0.21$) on UUN was found for the low protein diets, probably because of the low variation in UUN for the low protein diets. This means that an increase in excretion of urea is carried out by (1) an increase of the GFR, and (2) by a reduction of urea recycling from the glomerular filtrate. These 2 UUN regulation mechanisms were also found in other studies (Schmidt-Nielsen and Osaki, 1958; Schmidt-Nielsen et al., 1958; Eriksson and Valtonen, 1982).

Urea Transfer to the Gastrointestinal Tract

The fact that K_{urea} was negatively related to dietary protein content was unexpected because it was hypothesized that an increase in UER, due to an increase in dietary protein intake and a subsequent increase in absorption of rumen NH_3 in the blood followed by an increase in urea synthesis by the liver, would increase the dilution rate of PUP. Probably, the 68% larger PUP for the high protein diets (47.3 g of urea-N) compared with the low protein diets (28.1 g of urea-N) was responsible for this lower K_{urea} . The negative effect of dietary NaCl on UER ($P = 0.020$) cannot be explained by a reduced NH_3 flux to blood plasma as a result of the negative

effect of NaCl on rumen NH_3 concentration (due to an increase in water intake resulting in an increase of rumen fluid passage rate and a reduction in fermentation of dietary protein and a dilution of rumen NH_3) as we observed no significant effect of dietary NaCl on rumen concentration of NH_3 . The fraction of UER returned to the gastrointestinal tract (GER:UER ratio) was negatively related to dietary protein content (Table 5). This negative relationship between dietary protein content and GER:UER ratio was also described by Reynolds and Kristensen (2008), although the observed ratios for the low and high protein diets in this study were higher compared with those predicted by Reynolds and Kristensen (2008) as shown in Figure 4. This difference was likely caused by an underestimated UUN in the present study. According to prediction equations presented by Kebreab et al. (2010), an average UN of 155 and 204 g of N/d would be expected for the low and high protein diets, respectively. These estimates from Kebreab et al. (2010) are 82 and 75 g of N/d higher than observed in this study for the low and high protein diets, respectively. Moreover, the high positive N-balance observed in this study cannot be explained by N-retention because the cows did not gain weight during the study. It remains unclear in what forms and via which routes this unaccounted N was lost because leakage of urine from the catheters was negligible and urine was acidified to a pH < 3.0 , supposed to be sufficient to prevent emission of NH_3 . However, when assuming that this unaccounted N originated from urinary urea and attributing the positive N-balance largely to UUN, the estimated GER:UER ratio for the

Figure 4. Relationship between dietary CP and the fraction of plasma urea entry rate (UER) that is returned to the gastrointestinal tract (GIT) in this study for high and low protein diets and in a previous study (Spek et al., 2012). Solid line represents the relationship derived by Reynolds and Kristensen (2008) based on 5 experiments with cattle using dual-labeled [^{15}N]urea infusion studies.

high protein diet would come close to predicted results reported by Reynolds and Kristensen (2008).

An effect of dietary NaCl content on the GER:UER ratio was expected, because of the negative effect of dietary NaCl on PUN and a lower urea concentration gradient between plasma and the rumen (wall); however, no such effect was observed in the present study (Table 5, Figure 4) or in an earlier study by Spek et al. (2012; unpublished results).

CONCLUSIONS

In this study, MUN, PUN, and UUN were positively related to dietary protein content, whereas the GER:UER ratio was negatively related to dietary protein content. Both MUN and PUN were negatively related to dietary NaCl content but UUN and GER:UER were not affected. No interaction was found between dietary protein and NaCl on MUN, UUN, renal parameters, UER, GER, and GER:UER ratio. The relationship between MUN and UUN was significantly affected by dietary NaCl. We conclude that for a proper interpretation of the relationship between MUN and UUN the effect of dietary salt should be considered.

ACKNOWLEDGMENTS

This study was funded by the Dutch Dairy Board (PZ, Zoetermeer, the Netherlands), the Dutch Product Board Animal Feed (PDV, Zoetermeer, the Netherlands), and the Dutch Ministry of Economic affairs, The Hague, the Netherlands).

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