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Differences between Holstein dairy cows in renal clearance rate of urea affect milk urea concentration and the relationship between milk urea and urinary nitrogen excretion

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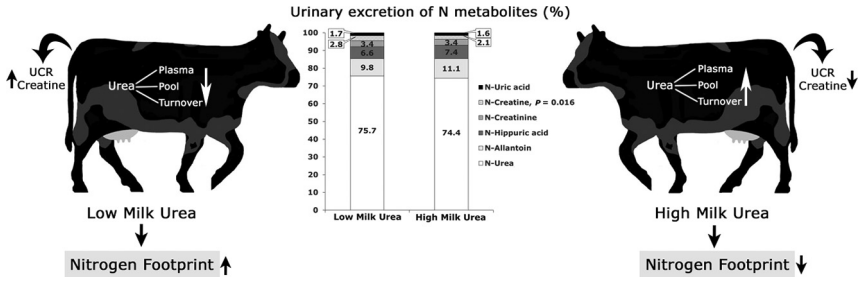
HIGHLIGHTS

- Reduction of the dietary crude protein content reduces urinary nitrogen excretion.
- Milk urea concentration is not sufficient to predict urinary urea emissions.
- Cows with high milk urea content have slower renal urea clearance rates, but
- … recycle more urea into the rumen, excrete less urinary creatine and
- … therefore have a lower environmental nitrogen footprint

GRAPHICAL ABSTRACT



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ABSTRACT

Urine and fecal excretions from cattle contribute to global nitrogen (N) emissions. The milk urea nitrogen (MUN) concentration in dairy cows is positively correlated with urinary urea N (UUN) emissions, and both decline with the reduction in crude protein intake. However, MUN concentration may differ between individual cows despite feeding the same ration. Thus, we hypothesized that due to differences in endogenous N utilization cows with high MUN concentration excrete more UUN than cows with a low MUN concentration. The objective of the present study was to elucidate N partitioning and urea metabolism in dairy cows with divergent MUN concentrations fed two planes of crude protein. Twenty Holstein dairy cows with high (HMU; $n = 10$) and low (LMU; $n = 10$) milk urea concentrations were fed two isocaloric diets with a low (LP) and normal (NP) crude protein level. Methane and ammonia emissions were recorded in respiration chambers. Feed intake, feces and urine excretions and milk yield were recorded for four days and subsamples were analyzed for total N and N-metabolites. A carbon-13 labeled urea bolus was administered intravenously followed by a series of plasma samplings. Total N and UUN excretions and ammonia emissions from excreta were lower on the LP diet, however, methane emissions, urinary N excretions and ammonia emissions were comparable between groups. Although plasma and salivary urea concentrations, urea pool size and urea turnover were higher, HMU cows had lower renal urea clearance rates. Additionally, HMU cows had lower renal clearance rates for creatinine, uric acid and creatine and excreted less uric acid (on the LP diet only) and creatine with urine. In conclusion, contrary to our hypothesis, HMU cows did not excrete more UUN than LMU cows. The lower urinary creatine excretion of HMU cows suggests that these animals have a lower environmental nitrogen footprint.

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1. Introduction

Anthropogenic N emissions are a growing worldwide problem contributing to particulate matter in the atmosphere, greenhouse gas emissions, eutrophication of soil and water, all of which are associated with negative consequences for human, animal and environmental health (Leip et al., 2015). Livestock farming is responsible for 76% of the global N emissions (Leip et al., 2015) with cattle husbandry being a major source accounting for 36% of ammonia emissions in Europe (Rösemann et al., 2019). Major ammonia emissions from livestock farming originate from urinary urea hydrolysis, which is catalyzed by microbial urease excreted with feces (Muck and Steenhuis, 1982). The dietary CP intake is the major factor determining the production of ruminal ammonia, which is absorbed, detoxified by the liver of the host yielding urea. The latter is subsequently excreted predominantly via urine (Lapierre and Lobley, 2001). Thus, the level of CP intake determines urinary urea N (UUN) excretions and thereby ammonia emission from manure (Burgos et al., 2007; Mutsvangwa et al., 2016). For dairy cows, milk urea concentration may serve as an indicator for monitoring an optimal CP content and protein/energy ratio in the diet. Under practical conditions, 1% change in dietary CP content results in a 11 mg/L change in MUN concentration of Holstein and Jersey x Holstein crossbreeds (Aguilar et al., 2012). The MUN is positively correlated with urinary N (Ciszuk and Gebregziabher, 1994; Jonker et al., 1998) and UUN excretions (Burgos et al., 2007), and thereby directly related to ammonia emissions from the manure of dairy cows (van Duinkerken et al., 2005; Burgos et al., 2010) and environmental burden. Further dietary factors such as the energy, concentrate, RUP, and NDF contents (Broderick, 2003; Hojman et al., 2004; Edouard et al., 2016), or the level of sodium intake (Spek et al., 2013a) may influence the linear relationship between MUN and UUN. Nevertheless, the dietary CP level is the main factor influencing UUN, and reduction of the CP content is one key for mitigating N emissions from dairy farms (Hristov et al., 2011). A reduction in CP intake leads to decreased urea concentrations in plasma and milk and improves the N efficiency of microbial protein synthesis by increasing the urea transport into the rumen (Lapierre and Lobley, 2001; Kohn et al., 2005). Moreover, UUN excretion is reduced by a lowered renal urea clearance rate and increased renal urea reabsorption rate, both contributing to the reuse of urea for microbial protein synthesis in the rumen (Spek et al., 2013b). However, depending on the further diet composition, reducing the dietary N content while increasing the fiber content may result in an unwanted decrease in milk yield and increased methane emissions (Bannink et al., 2010; Acharya et al., 2015; Niu et al., 2016). Apart from dietary factors, animal-related traits such as BW has been described to be positively associated with urinary N excretion (Kauffman and St-Pierre, 2001) or negatively with MUN concentrations (Hojman et al., 2005). Moreover, Johnson and Young (2003) and Hojman et al. (2005) reported lower MUN concentrations in first compared to higher lactating cows. Aguilar et al. (2012) showed clear phenotypic differences in MUN neither basing on dietary differences nor differences in milk performance, BW or stage of lactation. A moderate heritability for MUN was reported, suggesting that MUN might be utilized for selecting cows with reduced N excretions (Beatson et al., 2019). In fact, milk urea concentrations of dairy cows receiving the same feeding ration and producing comparable amounts of milk ranged between 150 and 300 mg/L, as tested on the same day (Richardt et al., 2002). This observation let us hypothesize that dairy cows with an intrinsically low milk urea concentration excrete less urinary urea than cows with high milk urea concentrations, and that these differences can be attributed to a different N partitioning between milk, feces and urine. Inter-individual differences in the partitioning of N metabolites may involve differences in renal urea clearance and reabsorption, urea transfer to the gastrointestinal tract and urea hydrolysis. Therefore, the aim of the present study was to elucidate N excretions via milk, urine and feces and physiological mechanisms regulating urea metabolism in dairy cows with divergent milk urea concentration fed two dietary CP levels.

2. Materials and methods

2.1. Animals

The experimental procedure was approved by the state office of Agriculture, Food Security and Fishery Mecklenburg-Western Pomerania, Rostock, Germany (LALLF 7221.3–1-052/17). Twenty multiparous German Holstein cows were bought in pairs from commercial farms in Mecklenburg-Western Pomerania. Each pair was on the same diet in commercial farms and included one cow with a higher (HMU: 277 ± 9 mg/L; $n = 10$) and lower (LMU: 189 ± 12 mg/L; n = 10) milk urea concentration based on the last five monthly milk test recordings. Cow pairs were transported to the institutional barn and adapted to a loose housing system with free access to water and feed for two weeks. At the start of the experiment, cows were 301 ± 5 DIM in second to fourth lactation, not pregnant, had a milk yield of 34.1 ± 1.1 kg/d and a BCS of 3.4 ± 0.1 on a five-point scale. The first six cow pairs were fed two isocaloric diets (10.1 ± 0.2 MJ metabolizable energy/kg DM) with two different CP levels (low protein: LP = 13.8 ± 0.2%; normal protein: NP = 15.9 ± 0.1%) in a crossover design separated by a two-week washout period. The remaining four pairs received either the LP or NP diet only, resulting in four experimental groups HMU-LP, HMU-NP, LMU-LP and LMU-NP (each $n = 8$). Diets were offered twice-daily 0500 h and 1700 h for ad libitum intake as total mixed ration (TMR) (Table 1) after milking at 0430 h and 1630 h. The backfat thickness was measured sonographically (Schroeder and Staufenbiel, 2006) at the beginning of the experimental procedure.

2.2. Methane and ammonia emissions, heat production and energy corrected milk

After the adaptation or washout period, cows were transferred to the institute's open-circuit respiration chambers (Derno et al., 2009) to which they were at least 3-times adapted to before. Cows were considered adapted when they consumed feed, drunk water, laid down and ruminated. The gas recovery rate of the chambers was 99.9 ± 0.96%. Chambers were climate controlled (15.5–16.5 °C, 70% humidity) and the airflow adjusted to 30 m^3/h. After a 12-h gas equilibration period, O_2, CO_2, CH_4 and NH_3 gas concentrations were recorded over a period of 48 h. Gas samples were drawn by a membrane pump (80 L/h; KNF Neuburger Laboport, Freiburg, Germany) and analyzed by a paramagnetical analyzer for O_2 and by infrared absorption (NDIR) for CO_2, CH_4 and NH_3 concentrations (SIDOR and GMS800; SICK AG, Reute, Germany) in 6 min intervals. Heat production (HP) was calculated per unit metabolic BW (mBW) according to Brouwer (1965):

$$\mathrm{HP/mBW}\left(\mathrm{kJ/kg^{0.75}}\right) = [16.18 \times \mathrm{O_2\ (L)} + 5.02 \times \mathrm{CO_2\ (L)} - 2.17 \times \mathrm{CH_4\ (L)} - 5.99 \times \mathrm{N_{Urine}\ (g/d)}]/\mathrm{mBW}\left(\mathrm{kg^{0.75}}\right),$$

where, daily urinary N (N_{Urine}) excretion was determined in pooled acidified urine samples as described in Section 2.3. Feeding, milking and cleaning in the chambers were realized via an air lock flushed with chamber air at 0630 h and 1630 h. Milk aliquots were taken at each milking and sent to a routine laboratory (Milk Testing Services North Rhine-Westphalia, Krefeld, Germany) for the analysis of total protein, fat, lactose and urea by mid-infrared spectroscopy (MilkoScan, Foss GmbH, Rellingen, Germany) and somatic cell content by flow cytometry (Fossomatic, Foss GmbH, Rellingen, Germany). The energy corrected milk (ECM) yield was calculated from milk composition according to: ECM (kg/d) = [0.038 × fat (g) + 0.024 × protein (g) + 0.017 × lactose (g)] × milk (kg/d) / 3.14. Before and after transfer to the chambers, BW was recorded to calculate the average.

Table 1
Feed constitutes, nutrient composition and energy concentration of the normal protein (NP) and low protein (LP) diets (means ± SEM).

Component	NP	LP
Ingredients, g/kg of DM		
Grass silage	309 ± 2	273 ± 3
Corn silage	330 ± 10	408 ± 7
Hay	15 ± 7	–
Barley straw	–	4.9 ± 3
Corn meal	72 ± 5	97 ± 7
Wheat seeds	104 ± 8	109 ± 4
Rapeseed extraction meal	156 ± 4	93 ± 5
Mineral feed[a]	9.0 ± 0.1	11.0 ± 0.2
Limestone[b]	3.3 ± 0.1	3.4 ± 0.2
Feed salt[c]	1.7 ± 0.0	1.5 ± 0.0
Nutrients, g/kg of DM		
Crude ash[d]	76 ± 3	76 ± 6
Crude fat	31 ± 1	29 ± 1
Crude protein	159 ± 1	138 ± 2
Crude fiber	177 ± 3	164 ± 3
ADF	199 ± 3	189 ± 3
NDF	382 ± 6	356 ± 5
Starch	205 ± 5	249 ± 4
Sugar	20 ± 1	12 ± 2
ME, MJ/kg DM	10.1 ± 0.2	10.2 ± 0.3
NE_L, MJ/kg DM	6.1 ± 0.2	6.2 ± 0.2
Utilizable crude protein[e]	142 ± 2	138 ± 3
Ruminal N balance[f]	2.7 ± 0.4	−0.1 ± 0.4
CP/ME, g/MJ	15.9 ± 0.4	13.7 ± 0.4
N, g/kg DM[g]	29.5 ± 0.4	25.2 ± 0.2

[a] Panto Mineral R 8609 (HL Hamburger Leistungsfutter GmbH, Hamburg, Germany): composition: 20% calcium, 6% phosphorous, 8% sodium, 6% magnesium, 0.03% inorganic nitrogen, 13.74% phosphorous pentoxide. Additives per kg original substance: 900,000 IU vitamin A, 200,000 IU vitamin D_3, 4.5 g vitamin E, 1.5 g Cu, 8 g Zn, 5 g Mn, 60 mg I, 21 mg Co, 50 mg Se.
[b] Bergophor $CaCO_3$ V001 (Hohburg Mineralfutter GmbH, Lossatal, Germany): 37% calcium.
[c] Animal feed salt (ESCO - European Salt Company GmbH & Co.KG, Hanover, Germany): 38% sodium, 0.3% calcium, 0.01% magnesium.
[d] Measured quantity elements g/kg in LP: calcium 7.0 ± 0.2, phosphorous 4.1 ± 0.1, sodium 2.3 ± 0.2, magnesium 2.3 ± 0.1, potassium 10.3 ± 0.6; NP: calcium 7.5 ± 0.4, phosphorous 4.4 ± 0.1, sodium 2.4 ± 0.2, magnesium 2.6 ± 0.1, potassium 10.5 ± 0.6.
[e] Utilizable crude protein (g/kg DM) = [11.93 – (6.82 × UDP) (g/kg DM) / crude protein (g/kg DM)] × ME (MJ/kg DM) + 1.03 × UDP (g/kg DM), with UDP = undegradable protein (GfE, 2001).
[f] Ruminal N balance (g/kg DM) = [crude protein (g/kg DM) – utilizable crude protein (g/kg DM)] / 6.25 (GfE, 2001).
[g] N measured in fresh feed including volatile nitrogen compounds and normalized to dry matter content.

2.3. Nitrogen balance, carbon-13 labeled urea tracer and samplings

After leaving the respiration chambers, cows were transferred to tie stalls on a 2.43 × 1.56 m stand with rubber mats at 15 °C (day 1). Each ration (Table 1) was prepared in one batch to reduce between-day variations, sampled (approx. 2 kg), vacuum-packed in 40-kg plastic bags and stored at 4 °C before feeding. During the first 5 days, cows were fed ad libitum and feed intake as well as water intake was recorded to calculate the daily mean. Starting with the morning feeding on day 6 to day 9, 95% of the daily ad libitum intake was provided in 8 meals. The first 7 meals amounting to 56% of the daily ad libitum intake were provided in equal portions from 6000 h to 1800 h in 2 h intervals, while the remaining 39% was given in one portion at 2000 h. Cows were equipped with a urinal consisting of a fabric funnel with a velcro pad covering the vulva area (Kauffman and St-Pierre, 2001). On day 5, the urinal funnel was connected to a flexible tube (4.5 cm, inner diameter) leading to a 30-L plastic container for quantifying weight and volume of urine released within 24 h and determining the urine density. Non-acidified urine collected on day 5 was sampled to measure non-urea urine nitrogen (non-UUN) concentrations (Section 2.4.3). During interval feeding (day 6–9), the container was pre-filled daily with 409 mL (562 g) 50% sulphuric acid to prevent urinary urea degradation and volatile N-losses during urine collection. The container was placed on a shaker or magnetic stirrer for immediate mixing of acid with urine. After 24 h, the weight and volume of the acidified urine were determined, the pH was measured confirming <2.0, and aliquots were taken and stored at −20 °C before analyses. Recorded urine volume and analytics were corrected for added sulphuric acid. On day 5 and during interval feeding, milk yield was measured after milking at 0630 h and 1830 h and 15-mL milk aliquots were frozen at −20 °C. Feces were collected several times daily and transferred to 4 °C storage to minimize volatile N losses. The 24-h collections were weighted, stirred and subsamples were stored at −20 °C. On day 8, the mouth was rinsed with water and after 10 min, one saliva sample was collected using a sponge. Saliva was centrifuged (2951 ×*g*, 10 min, 4 °C) and frozen at −20 °C. On day 1 before and on day 5 after first meal, a 750-mL rumen fluid sample was taken using an esophageal probe connected to a vacuum pump to measure pH and ammonia concentration. On day 5, cows received a jugular vein catheter (Cavafix Certo m. Splittocan, 32 cm length; B. Braun Melsungen AG, Melsungen, Germany) flushed with 0.9% saline. On day 7, two blood samples were taken from the catheter −10 and −5 min relative to tracer administration to measure the mean natural ^{13}C urea abundance. The tracer (^{13}C urea; ≥99 atom%; Sigma-Aldrich, St. Louis, USA) was administered as a bolus (2 g per 650 kg average BW, dissolved in 0.9% saline) and the catheter was flushed with 20 mL 0.9% saline. A series of blood samples was taken in EDTA-containing 9-mL tubes (S-Monovetten; Sarstedt, Nümbrecht, Germany) at 5, 10, 20, 30, 60, 120, 180, 240, 360, 480, 600 and 1320 min after bolus administration to allow for the modeling of exponential ^{13}C urea kinetics (Section 2.4.5). Blood samples were immediately placed on ice and a 1-mL aliquot was frozen at −20 °C. The remaining blood was centrifuged (1345 ×*g*, 20 min, 4 °C) and obtained plasma stored at −20 °C.

2.4. Analytics

2.4.1. Feed nutrients

The DM of feed was determined after air-drying at 60 °C, grinding and repeated drying at 105 °C for 4 h. Nutrient composition was analyzed by the "Landwirtschaftliche Untersuchungs- und Forschungsanstalt, Rostock" (LUFA GmbH, Rostock, Germany) (Table 1). The Weender analysis included crude fat (Soxhlet petrol ether extraction without acidification), CP (Kjeldahl N-determination, CP = N x 6.38) and crude fiber (sulphuric acid and sodium hydroxide treatments) and crude ash (heating at 475–550 °C till constant weight) analysis. The van Soest analysis involved quantification of sugar (Luff-Schoorl method, reduction of Fehling's solution), starch (optical rotation after treatment with hydrochloric acid and ethanol), ADFom (treatment with sulphuric cetyltrimethyl-ammonium bromide solution) and aNDFom (incubation with amylase).

2.4.2. Gross and net energy

Feed and feces samples were dried as described in 2.4.1 and urine samples were freeze-dried before subjected to bomb calorimetrically (IKA C 7000, IKA-Werke GmbH & Co. KG, Staufen, Germany; in feed, feed residues and faces heat of combustion measured in duplicates, in freeze-dried urine samples as quadruple determination) to measure gross energy (GE). As 3 urine samples were not dryable, their GE content was calculated using the caloric value of urea (10.57 kJ/g), uric acid (11.46 kJ/g), allantoin (10.51 kJ/g) and hippuric acid (23.66 kJ/g) (Nehring, 1972). Based on the caloric value of methane (55.87 kJ/g; Nehring (1972)), the metabolizable energy (ME) was calculated as: ME (MJ/kg) = $GE_{Feed} - GE_{Urine} - GE_{Feces} - GE_{CH4}$. The net energy for lactation (NE_L) was calculated according to GfE (2001): NE_L (MJ/kg) = 0.6 × [1 + 0.004 × (q – 57)] × ME (MJ/kg).

2.4.3. Total nitrogen analysis

Frozen feed was grinded on dry ice and drinking water sampled into a glass bottle. Feces (day 6–7), milk (day 6–8) and acidified urine samples (day 6–8) were proportionally pooled according to the amount

excreted within 24 h. All samples were sent to the LUFA GmbH for total N analysis. Total N-content in fresh grinded feed and pooled feces was determined using the Kjeldahl method, and in milk and urine according to Dumas using a "vario MAX" element analyzer (Elementar; Langenselbold, Germany). Total N-content in drinking water was analyzed following the methods of the German Institute for Standardization (N-concentration = 0.58 mg/L). Subsequently, N-balance was calculated as the difference between N intake (NI) from feed and water and N excretions via urine, feces and milk. Apparent digestibility (AD) of N was calculated as AD (%) = [NI (g/d) – FN (g/d)] / NI (g/d) × 100, where, FN refers to fecal N-excretion, and N utilization efficiency (NUE) by dividing daily N milk excretion by daily N intake.

2.4.4. Constitutes and N-metabolites in milk, plasma, urine and saliva

For the analysis of N-metabolite concentrations, milk was centrifuged (50,000 ×*g*, 10 min, 4 °C) to obtain the aqueous phase. Creatinine, uric acid and urea concentrations in centrifuged milk and plasma samples obtained 60, 600 and 1320 min after tracer administration, as well as non-esterified fatty acids (NEFA) and triglycerides (TG) in plasma taken 60 min after tracer administration, and saliva urea and creatinine concentrations were analyzed spectrophotometrically and potentiometrically (ABX Pentra C400 clinical chemistry analyzer; HORIBA Europe GmbH, Oberursel, Germany) using the following kits: A11A01-907 (creatinine), -670 (uric acid), -641 (urea), -640 (triglycerides) (HORIBA ABX SAS, Montpellier, France) and 434–91,795/436–91,995 (NEFA) (FUJIFILM Wako Chemicals Europe GmbH, Neuss, Germany). Furthermore, pooled plasma samples obtained 60, 600 and 1320 min after tracer application were sent to a testing laboratory (MVZ Medical laboratory Bremen GmbH, Bremen, Germany) for analysis of creatine concentrations according to Wong (1971). Briefly, plasma samples were subjected to the α-naphtol-diaceyl reaction and the absorbance was measured at 530 nm. Urea, uric acid and creatinine concentrations measured in the aqueous phase of milk were calculated to whole milk considering milk fat and protein content. Plasma samples obtained 60 min after tracer administration were subjected to free AA analysis using HPLC with fluorescence detection (HPLC 1200 Series; Agilent Technologies, Waldbronn, Germany) as described earlier (Kuhla et al., 2010) installing a 250 × 4 mm HyperClone ODS (C18) 120 Å column (Phenomenex, Aschaffenburg, Germany). Urinary urea concentrations were analyzed from 50-fold diluted acidified samples, whereas, due to their instability under acidic conditions, urinary allantoin, creatinine, creatine, hippuric acid and uric acid concentrations were analyzed in tenfold diluted non-acidified samples obtained on day 5 using HPLC (1200/1260 infinity Series; Agilent Technologies). Urea concentrations were measured by passing a 300 × 7.8 mm Rezex RCM-Monosaccharide column (Phenomenex Inc.) protected by a 4 × 3 mm Carbo-Ca pre-column at 60 °C and detected by refractive index (RI). Ultrapure water was used as mobile phase at a flow rate of 0.5 mL/min (App-ID: 5518, Phenomenex Inc.). Non-urea N-metabolites were measured on a 250 × 4 mm HyperClone ODS (C18) 120 Å column protected by a 4 × 3 mm C18 pre-column at constant 25 °C. Phosphate buffer (20 mM, pH 6.5) was used as mobile phase at a flow rate of 1 mL/min (Yang, 1998). Metabolites were detected at 230 nm with exception for creatine at 210 nm. Non-UUN was calculated as the difference between urinary N and UUN.

The renal clearance rate for creatinine (CCR), urea (UCR), uric acid (UACR) and creatine (CREACR), respectively, was calculated according to:

$$\mathrm{CCR\ (L/min)} = \mathrm{Creatinine_{Urine}\ (mmol/d)/Creatinine_{Plasma}\ (mmol/L)} /1440\ (\mathrm{min/d});$$

$$\mathrm{UCR\ (L/min)} = \mathrm{Urea_{Urine}\ (mg/d)/Urea_{Plasma}\ (mg/L)/1440\ (min/d)};$$

$$\mathrm{UACR\ (L/min)} = \mathrm{UricAcid_{Urine}\ (mg/d)/UricAcid_{Plasma}\ (mg/L)/1440\ (min/d)};$$

$$\mathrm{CREACR\ (L/min)} = \mathrm{Creatine_{Urine}\ (mg/d)/Creatine_{Plasma}\ (mg/L)/1440\ (min/d)}.$$

The renal urea reabsorption ratio (RRR) was calculated as: RRR = 1 – (UCR / CCR) (Spek et al., 2013a).

2.4.5. Rumen fluid ammonia concentrations and fecal urease activity

Ammonia concentrations were measured according to the Conway method (Kenten, 1956). Briefly, 1 mL potash was added to 1 mL rumen fluid to release ammonia reacting with 5 mL reagent solution consisting of 5 g boric acid, 200 mL ethanol, 300 mL distilled water and 10 mL of an indicator solution (33 mg bromocresol green and 66 mg methyl red in 100 mL ethanol). After 24 h, the solution was titrated against 0.01 N hydrochloric acid to pH 7.0. Frozen fecal samples from a subset of 6 animals per group were thawed and pooled to determine fecal urease activity according to Dai and Karring (2014). Thereby, reaction mixtures, containing 10 g of thawed pooled feces, 23 mL distilled water and 3 mL of 400 mmol/L phosphate buffer (pH 7.0), were incubated with 4 mL urea solution (2 mol/L) at constant 25 °C, followed by measuring ammonia concentrations after 5 and 10 min according to the Conway method (Kenten, 1956). Measurements were repeated three times for each cow. Enzyme activities were averaged and normalized to fecal DM content.

2.4.6. Plasma ^{13}C urea and whole blood $^{13}CO_2$ enrichments

A 50 μL plasma sample was treated with 300 μL acetonitrile followed by a centrifugation (590 ×*g*, 10 min, 4 °C) for deproteinization. The supernatant was dried at 60 °C under a N_2 stream and incubated with 25 μL *N*-methyl-N-(*t*-butyldimethylsilyl)-trifluoroacetamide (MTBSTFA) in 25 μL pyridine. The mixture was vortexed for 1 min and kept at 105 °C for 30 min. Finally, 100 μL ethyl acetate was added and samples were stored at −20 °C until analysis on a gas chromatograph-mass spectrometer (GC–MS, QP 2010, coupled with GC 2010, AOC-20i; Shimadzu, Duisburg, Germany). The *t*-butyldimethylsilyl derivatives were detected at *m/z* 231 and 232, respectively. A two-exponential curve fitting to the ^{13}C urea enrichment calculated as mol % excess (MPE) was applied: $\mathrm{MPE}(t) = a \times e^{(-b \times t)} + c \times e^{(-d \times t)}$ using TableCurve 2D software (ver. 5.0.1; Systat GmbH, Erkrath, Germany), where a, b, c and d are the variables defining the ^{13}C urea enrichment curve (Fig. 1). Based on these variables, the area under the curve (AUC) was calculated as AUC = a/b + c/d. Considering the tracer dosage (D) in mg, the whole-body ^{13}C urea turnover rate (rate of appearance or urea entry rate (UER)), and urea pool size (Q) were calculated according to (Wolfe and Chinkes, 2005):

$$^{13}\mathrm{C\ urea\ turnover\ rate\ [mg/(d \times kg\ BW)]} = \mathrm{D/(AUC \times BW)\ [mg/(h \times kg\ BW)]} \times 24;$$

$$\mathrm{UER\ (g/d)} = {}^{13}\mathrm{C\ urea\ turnover\ rate\ [mg/(d \times kg\ BW)]} \times \mathrm{BW\ (kg)/1000};$$

$$\mathrm{Q\ (mg/kg\ BW)} = {}^{13}\mathrm{C\ urea\ turnover\ rate\ [mg/(d \times kg\ BW)]} \times \mathrm{MRT\ (h)/24},$$

with the mean residence time $\mathrm{MRT\ (h)} = (a/b^2 + c/d^2)/(a/b + c/d)$.

The fractional turnover rate (K_{Urea}) of ^{13}C urea from whole body was calculated as $K_{Urea}\ (h^{-1}) = {}^{13}\mathrm{C}$ urea turnover rate/(Q × 24), and the urea space was calculated as Q × kg BW/plasma urea (PU) concentration in mg/L. The urea degradation or gastrointestinal tract entrance rate (GER) was calculated according to (Spek et al., 2013a): GER (g urea-N/d) = UER (g urea-N/d) – UUN (g/d) – MUN (g/d) and the percentage of urea recycled was calculated as GER/UER × 100%.

The calculation of microbial urea oxidation from tracer enrichment in blood relies on the observation that urea is transferred into the rumen where it is converted by urease of ureolytic bacteria. The resulting CO_2 produced in the rumen is released with eructation or absorbed by the blood before exhalation. One milliliter whole blood

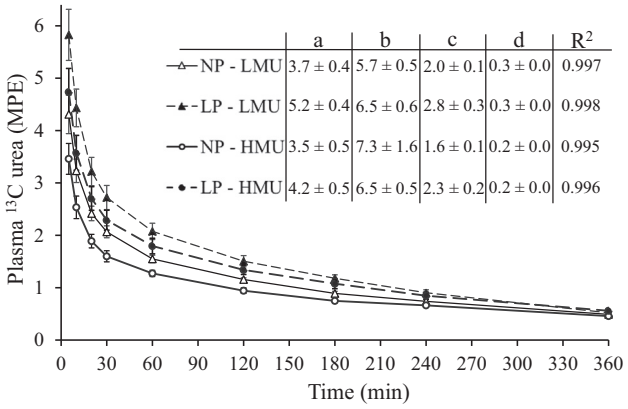

Fig. 1. Time course of ^{13}C urea enrichment in plasma until 360 min after i.v. ^{13}C urea tracer application in dairy cows grouped according to low (189 ± 12 mg/L; LMU) or high milk urea concentration (277 ± 9 mg/L; HMU) fed 15.8 ± 0.2% CP (normal protein; NP) or 13.7 ± 0.3% CP (low protein; LP). Data are shown as mean ± SEM. MPE = mole percent excess. The estimates for the two-exponential model for ^{13}C urea mol % excess (MPE): $MPE(t) = a \times \exp^{(-b\times t)} + c \times \exp^{(-d\times t)}$ and the corresponding coefficients of determination R^2 are shown in the legend.

samples were treated with 1 mL 10% lactic acid and the $^{13}CO_2/^{12}CO_2$ ratio was measured by an isotope ratio mass spectrometer (IRMS, DELTAplus XL; Thermo Quest, Bremen, Germany) coupled with a GasBench II (Finnigan, Bremen, Germany) and expressed as APE. An enrichment time curve (Junghans et al., 2007) was generated and submitted to best curve fitting (TableCurve 2D; Systat Software GmbH, Erkrath, Germany) for obtaining the area under the curve and calculating the average daily $^{13}CO_2/^{12}CO_2$ ratio. Subsequently, the urea hydrolysis rate (**UHR**) was calculated according to Slater et al. (2004):

$$\text{UHR}\ (\mu\text{mol/h}) = V\text{CO}_2\ (\text{mmol/30 min}) \times \text{whole blood}\ ^{13}\text{CO}_2\ \text{enrichment}_{30\,\text{min}}\ (\text{ppm excess}) \times 2/1000,$$

in which $V\text{CO}_2$ is the CO_2 production in 30 min after the morning feeding measured in respiratory chamber, and whole blood $^{13}CO_2$ $\text{enrichment}_{30\text{min}}$ is the $^{13}CO_2$ enrichment measured 30 min after ^{13}C urea administration.

2.5. Statistical analysis

One animal suffered from mild mastitis and one from fever (rectal temperature 39.3 °C for 1 day) during the experimental procedure, but showed no changes in feed intake or milk performance, and therefore were not excluded from statistical evaluation. Statistical analyses were performed using the SAS software for Windows, version 9.4 (Copyright, SAS Institute Inc., Cary, NC, USA). Data were analyzed by repeated measurement analyses of variance using the MIXED procedure. The ANOVA model contained the fixed factors milk urea concentration group (levels: HMU and LMU), diet (levels: NP and LP) and their interaction. Repeated measures on the same cow (repeated factor: diet) were taken into account by the REPEATED statement of the MIXED procedure using the SUBJECT = animal option to define the blocks of the block-diagonal residual covariance matrix and the TYPE = UN option to define their unstructured covariance structure. Least-squares means (LS-means) and their standard errors (SE) were computed for each fixed effect in the model, and all pairwise differences of these LS-means were tested by the Tukey-Kramer test, a procedure for pairwise multiple comparisons. In addition, the SLICE statement of the MIXED procedure was used for performing partitioned analyses of the LS-means for the two-way interaction milk urea concentration group*diet (i.e. test milk urea concentration groups within the levels of diet and test of the diets within the levels of milk urea concentration group). Correlations between UUN, MUN and PU were determined and tested using the CORR Procedure of Base SAS software. Data obtained are presented as LSM ± SE unless otherwise specified.

3. Results

3.1. Animal characteristics and gas exchange measurements

Based on the monthly milk recordings in commercial farms, HMU and LMU cow groups did not differ in milk protein and lactose concentrations but milk fat percentage was higher in HMU cows (4.3 vs. 3.8%; $P = 0.038$). Cows with divergent MUN were comparable in DIM, BW, backfat thickness, feed and water intake, independent of the diet (Table 2). Milk yield of HMU cows was 1.3 kg/d higher on the LP than NP diet ($P = 0.035$; group x diet interaction: $P = 0.004$); however, this difference did not exist for LMU cows. Milk constitutes were not related to dietary CP content or cow groups except for milk fat content, which was 0.7% higher in HMU than LMU cows on the NP diet ($P = 0.022$; group × diet interaction: $P = 0.022$). Furthermore, milk fat content in LMU cows increased 0.2% with decreasing CP level ($P = 0.013$). Daily MUN secretion was positively affected by CP level ($P < 0.001$) and was in average 37.8% higher in HMU cows compared to LMU cows on both diets. Gas exchange measurements revealed no differences in O_2 consumption, CO_2 and CH_4 production between groups and diets; however, HP normalized to mBW was 1.7% ($P = 0.054$) and ammonia emissions were 64% ($P = 0.003$) higher when animals were fed the NP relative to LP diet.

3.2. Nitrogen balance and N metabolite excretions

Cows fed the NP diet consumed in average 80 g/d (19.5%) more N than on the LP diet ($P = 0.032$; Table 3), while N losses via milk were not affected by diet. However, fecal N excretion ($P = 0.089$) and urinary N excretion ($P < 0.001$) in both groups were positively affected by CP content. As a result, cows on the NP diet were in a slightly positive (4.15 g/d), but on the LP diet in slightly negative N-balance. The NUE was negatively associated (30.7% vs. 36.0%; $P < 0.001$), while the AD of N increased by 3.1% with increasing dietary protein level (64.1 vs. 61.0%; $P = 0.009$). There was no effect of milk urea concentration on total N intake, total N losses via milk, urine and feces, N balance, or NUE. However, compared to HMU cows, LMU cows secreted 24% less milk urea on the NP ($P = 0.025$) but not LP diet, and secreted 27% less milk uric acid on the LP ($P = 0.063$) but not NP diet. While milk urea secretion was positively related ($P < 0.001$), milk uric acid secretion decreased with increasing CP level ($P = 0.003$). The excretion of urinary urea decreased by 36% ($P < 0.001$) and urinary creatine by 12% ($P = 0.045$) with reduced CP level. While urinary urea excretion was not different between groups, LMU cows excreted 33% more urinary creatine than HMU cows on both diets ($P = 0.016$), and excreted 45% more urinary uric acid on the LP diet ($P = 0.078$) with an interaction between diet and group ($P = 0.018$). Moreover, the excretion of creatine relative to total urinary N was also higher in LMU cows, whereas the proportional excretion of other N-metabolites was not different between groups (Fig. 2). Total urinary allantoin, creatinine, hippuric acid and non-UUN excretions were not affected by dietary treatment or group, whereas the UUN and MUN excretion ratio (UUN/MUN) was 1.4-fold greater in LMU than HMU cows ($P = 0.032$), irrespective of diet (Table 3). Furthermore, there was a substantial correlation between UUN and MUN excretions including both diets and groups ($r = 0.479$; $P = 0.006$), but when separated by diet, UUN excretions and MUN secretions correlated positively for the LP ($r = 0.637$; $P = 0.008$), but negatively for the NP diet ($r = -0.428$; $P = 0.098$). However, the range in UUN was greater in LMU (50.2 to 160.2 g/d) than HMU (61.4 to 146.9 g/d) cows, whereas the MUN range was smaller in LMU (1.7 and 5.6 g/d) compared to HMU cows (2.3 to 6.9 g/d).

Table 2
Animal characteristics, energy intake, output and balance assessed during indirect calorimetry measurement of dairy cows with high (HMU) and low milk urea concentration (LMU) fed ad libitum a diet containing normal (NP) and low (LP) crude protein content. Data are given as least square means and standard error (SE).

Parameter[1]	NP				LP				*P*-value[2]		
	HMU	LMU	SE	*P*-value[3]	HMU	LMU	SE	*P*-value[3]	Diet	Group	Diet × group
DIM	303	291	11	0.473	292	283	11	0.589	0.366	0.414	0.889
BW, kg	655	681	19	0.358	655	674	21	0.533	0.588	0.425	0.555
BCS	3.4	3.7	0.3	0.610	3.2	3.4	0.2	0.565	0.335	0.458	0.895
Backfat, mm	13	15	4	0.818	10	13	2	0.398	0.611	0.445	0.885
DMI, kg/d	17.0	17.5	0.9	0.710	18.1	17.1	0.8	0.376	0.436	0.829	0.082
MEI, MJ/d	171	171	11	0.994	184	170	11	0.423	0.499	0.615	0.400
Water intake, kg/d	71	68	4	0.655	69	64	5	0.475	0.194	0.533	0.523
Milk yield, L/d	23.4[b]	23.9[ab]	1.7	0.859	24.7[a]	23.2[ab]	1.6	0.510	0.398	0.826	0.005
ECM, kg/d	23.4	21.9	1.4	0.447	24.2	21.5	1.2	0.134	0.759	0.252	0.377
Milk fat, %	4.7[a]	4.0[b]	0.2	0.022	4.7[ab]	4.2[a]	0.2	0.073	0.189	0.044	0.022
Milk protein, %	3.8	3.6	0.2	0.593	3.7	3.5	0.1	0.509	0.618	0.279	0.992
MUN, g/d	1.93[a]	1.35[b]	0.1	0.002	1.39[b]	1.06[a]	0.1	0.013	<0.001	<0.001	0.194
Milk lactose, %	4.9	4.7	0.1	0.208	4.8	4.7	0.1	0.454	0.865	0.241	0.216
SCC, ×1000 cells/mL	104	521	182	0.112	682	605	397	0.893	0.172	0.651	0.304
Oxygen, g/d	9088	9066	298	0.965	8849	8893	298	0.930	0.473	0.976	0.894
Carbon dioxide, g/d	13,151	13,055	523	0.919	13,161	13,084	495	0.930	0.967	0.891	0.985
Methane, g/d	382	388	15	0.778	379	384	17	0.839	0.641	0.795	0.938
Ammonia, g/d	7.4[a]	6.9[ab]	1.0	0.762	4.1[b]	4.6[ab]	0.6	0.578	0.003	0.984	0.562
HP/mBW, kJ/kg$^{0.75}$	1075	1055	38	0.713	1056	1039	32	0.705	0.054	0.709	0.888

[a,b]Different superscript letters within one row indicate $P < 0.05$ (Tukey test).
[1] ECM = energy corrected milk yield; MEI = metabolizable energy intake; MUN = daily milk urea N secretion; SCC = somatic cell content.
[2] *P*-value from ANOVA analysis.
[3] *P*-value from Tukey slice test.

3.3. Plasma metabolite concentrations

PU concentrations were 30% higher with NP than LP feeding ($P < 0.001$), and was lower in LMU than HMU cows ($P = 0.059$; Table 4). There was a significant correlation between PU and MUN concentrations including all cows and diets ($r = 0.850$; $P < 0.001$). Plasma uric acid concentrations were 15% lower in LMU than HMU cows on the LP diet ($P = 0.078$), whereas plasma creatinine concentrations were in average 11% higher in HMU than LMU cows ($P = 0.035$). Plasma creatine concentrations were 4 to 11% higher ($P < 0.05$) and plasma citrulline (Cit) concentrations 28% higher in HMU than LMU cows ($P = 0.085$). Plasma glycine (Gly), methylhistidine and serine (Ser) concentrations ($P < 0.05$) increased by 12% with the reduction in dietary CP level. Interestingly, plasma glutamine (Gln) ($P = 0.021$; group × diet interaction: $P = 0.019$), and α-aminobutyric acid concentrations increased with the reduction in dietary protein level in LMU cows ($P = 0.002$; group × diet

Table 3
Nitrogen intake, excretions, balance, utilization efficiency and apparent digestibility of dairy cows with low (LMU) and high milk urea concentration (HMU) fed normal (NP) and low (LP) dietary crude protein level under conditions of feeding 95% of the ad libitum intake. Data are given as least square means and standard error (SE).

Parameter[1]	NP				LP				*P*-value[2]		
	HMU	LMU	SE	*P*-value[3]	HMU	LMU	SE	*P*-value[3]	Diet	Group	Diet × group
Total N measures											
N-intake, g/d	498	483	25	0.707	416	405	19	0.711	0.032	0.632	0.908
N-milk, g/d	157	143	11	0.409	154	142	9	0.377	0.697	0.335	0.904
N-urine, g/d	159[ac]	162[ab]	6	0.845	113[bd]	115[cd]	8	0.745	<0.001	0.729	0.967
N-feces, g/d	180	173	10	0.669	164	159	7	0.596	0.089	0.549	0.963
N-balance, g/d	4.9	3.4	14.1	0.765	−12.8	−7.8	11.8	0.940	0.199	0.909	0.767
NUE, %	31.7[cd]	29.6[bd]	1.4	0.311	36.8[ab]	35.2[ac]	1.8	0.552	<0.001	0.384	0.807
AD of N, %	64.4[a]	63.8[ab]	1.3	0.745	61.2[b]	60.8[ab]	1.2	0.859	0.009	0.765	0.877
Milk N metabolites, g/d											
Urea	11.0[a]	8.4[b]	0.8	0.025	7.1[bc]	5.9[ac]	0.6	0.167	<0.001	0.055	0.068
Creatinine	0.38	0.31	0.03	0.135	0.37	0.33	0.03	0.297	0.667	0.145	0.219
Uric acid	0.38[b]	0.30[ab]	0.05	0.256	0.45[a]	0.33[ab]	0.04	0.063	0.003	0.140	0.363
Urinary N metabolites, g/d											
Urea	273[ab]	273[ac]	12	0.964	168[cd]	182[bd]	14	0.494	<0.001	0.554	0.642
Allantoin	45	38	5	0.371	40	38	4	0.758	0.546	0.384	0.554
Creatine	9.3[b]	13.4[a]	0.9	0.003	9.0[ab]	11.0[b]	1.0	0.155	0.045	0.016	0.109
Creatinine	12.3	12.6	0.7	0.781	12.3	13.5	0.7	0.245	0.399	0.383	0.431
Hippuric acid	138	114	11	0.180	122	124	10	0.866	0.790	0.375	0.238
Uric acid	6.7[ab]	6.2[b]	0.7	0.607	6.0[ab]	8.1[a]	0.8	0.078	0.233	0.412	0.018
Non-UUN, g/d	31.6	34.2	2.2	0.422	33.9	29.0	3.6	0.357	0.594	0.737	0.188
UUN, g/d	127[ab]	128[ac]	6	0.964	78[cd]	85[bd]	7	0.494	<0.001	0.554	0.642
UUN/MUN	26[ab]	36[ab]	4	0.105	24[b]	32[a]	2	0.009	0.286	0.032	0.680

[a-d]Different superscript letters within one row indicate $P < 0.05$ (Tukey test).
[1] AD = apparent digestibility; MUN = milk urea N secretion; NUE, N-utilization efficiency = N-milk (g/d) / NI (g/d) × 100; non-UUN = non-urea urine N excretion; UUN = urinary urea N excretion.
[2] *P*-value from ANOVA analysis.
[3] *P*-value from Tukey slice test.

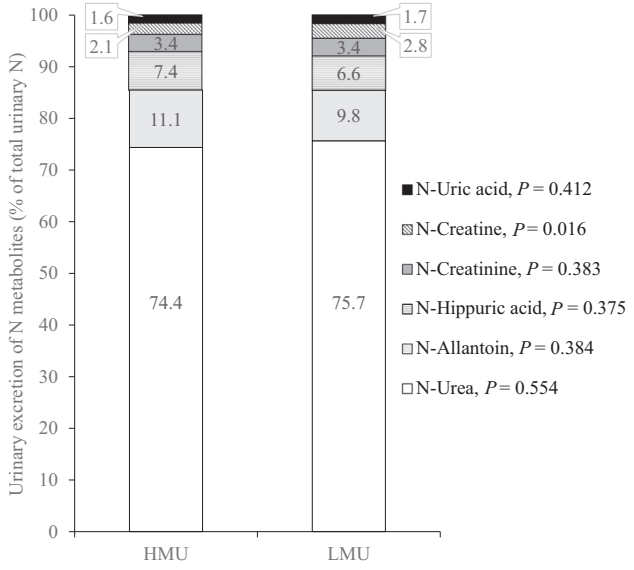

Fig. 2. Mean percentage of excreted urinary N metabolites of total urinary N in dairy cows with low (189 ± 12 mg/L; LMU) and high milk urea concentration (277 ± 9 mg/L; HMU) fed LP and NP rations. *P*-values from Tukey test.

interaction: $P = 0.015$), but were unchanged in HMU cows. The concentration of other proteinogenic amino acids (data not shown) and the urea cycle amino acid ornithine (Orn) (Table 4) were not different between diets and groups. Furthermore, LMU compared to HMU cows had 12% lower TG concentrations on the LP ($P = 0.044$) but not on the NP diet ($P = 0.897$; group × diet interaction: $P = 0.096$). Plasma NEFA concentrations did not differ between groups and diets.

3.4. Urea flow, recycling and kidney function

Evaluating the ^{13}C urea enrichments in plasma, we found a steeper decline for the NP compared to LP feeding, as well as in HMU than LMU cows (Fig. 1). Accordingly, the ^{13}C urea turnover rate was in average 24% higher on the NP than LP diet ($P = 0.004$) and 12% higher in HMU than LMU cows ($P = 0.084$; Table 5). These results were accompanied by a 41% larger urea pool size ($P < 0.001$) on the NP compared to LP diet and a 23% larger urea pool size in HMU than LMU cows ($P = 0.039$). Assuming that the urea appearance rate is equal to the urea disappearance rate, we next calculated the proportion of urea N released from the body pool. On average, the largest proportion of urea N (56.1%) was transferred into the rumen-intestinal tract, whereas 42.3% was excreted via urine and 1.6% secreted with milk. The proportion of urea N transferred into the gut increased by 11.8% with the reduction in dietary CP ($P < 0.001$). This effect was accompanied with proportionally lower urea N excretions via urine ($P = 0.026$) and milk ($P = 0.011$), lower salivary urea ($P = 0.014$) and ruminal ammonia ($P = 0.007$) concentrations, and a 45% higher UHR ($P = 0.008$), the latter reflected by the greater whole blood $^{13}CO_2$ enrichment with LP feeding ($P = 0.002$; Fig. 3). Fecal urease activity was not affected by diet. Furthermore, fecal urease activity, UHR and the portions of urea N transferred into different body compartments were not different between cow phenotypes, however, HMU cows had higher salivary urea concentrations than LMU cows (P = 0.011). While the RRR was higher ($P = 0.098$), the UCR was 18% lower ($P = 0.020$) when animals were fed the LP compared to NP diet. There were no significant differences in daily urine excretion and CCR between diets, but CCR was 0.21 L/min ($P = 0.049$; group: $P = 0.1$) and UACR 0.38 L/min ($P = 0.001$; group × diet interaction: $P = 0.033$) higher in LMU than HMU cows fed the LP diet. Both, CCR and UACR were negatively correlated with milk urea concentration ($r = -0.409$, $P = 0.020$ and $r = -0.340$, $P = 0.050$, respectively) including both diets, while UCR was negatively correlated with milk urea concentration only for the NP diet ($r = -0.677$, $P = 0.004$). The CREACR increased with CP level ($P = 0.011$) and was greater in LMU compared to HMU cows (group: $P = 0.004$; group × diet interaction: $P = 0.018$).

4. Discussion

4.1. Diet, milk composition, and gaseous emissions

Although the dietary CP level differed by an absolute value of 2.1% between the LP and NP diet, DMI, ECM, milk fat, protein and lactose percentages of cows were not affected by CP content, likely because the utilizable protein content was comparable between diets. In accordance with our findings, Niu et al. (2016) and Leonardi et al. (2003) found also no effect on DMI or ECM when CP levels were reduced from 18.5 to 15.2% or 18.9 to 16.1% in DM, respectively. Other studies, however, reported increases in DMI, milk performance, milk fat and protein yields when the CP level increased from 13.1 to 17.0% (Frank and Swensson, 2002) or from 14.3 to 16.3% (Acharya et al., 2015) on the DM basis. The improved milk performance reported earlier (Frank and Swensson, 2002; Acharya et al.,

Table 4
Plasma metabolite concentrations of dairy cows with low (LMU) and high milk urea concentration (HMU) fed normal (NP) and low (LP) dietary crude protein level under conditions of feeding 95% of the ad libitum intake. Data are given as least square means and standard error (SE).

Parameter	NP				LP				*P*-value[2]		
	HMU	LMU	SE	*P*-value[3]	HMU	LMU	SE	*P*-value[3]	Diet	Group	Diet × group
Urea, μmol/L	5225[a]	4317[b]	270	0.028	3800[bc]	3455[ac]	263	0.365	<0.001	0.059	0.212
Creatinine, μmol/L	86	78	4	0.206	91	80	4	0.132	0.284	0.035	0.653
Creatine, μmol/L	250	222	8	0.251	249	237	9	0.514	0.474	0.085	0.429
Uric acid, μmol/L	33	32	4	0.877	33	28	2	0.078	0.581	0.431	0.519
α-Aminobutyric acid, μmol/L	20[ab]	19[b]	2	0.571	20[ab]	22[a]	2	0.570	0.027	0.987	0.015
Arginine, μmol/L	98	101	9	0.798	87	93	6	0.537	0.256	0.577	0.895
Citrulline, μmol/L	102	77	7	0.109	93	77	6	0.210	0.456	0.020	0.447
Glutamine, μmol/L	361[ab]	314[b]	21	0.142	333[ab]	377[a]	21	0.165	0.322	0.969	0.019
Glycine, μmol/L	295[ab]	281[b]	17	0.538	305[ab]	332[a]	21	0.374	0.009	0.811	0.061
Methylhistidine, μmol/L	14.8[ab]	12.9[b]	0.7	0.093	16.1[ab]	15.3[a]	1.0	0.604	0.018	0.224	0.419
Ornithine, μmol/L	45	48	4	0.648	42	44	4	0.738	0.324	0.604	0.853
Serine, μmol/L	88[ab]	83[b]	5	0.516	92[ab]	101[a]	5	0.240	0.031	0.694	0.140
NEFA[1], μmol/L	119	89	22	0.374	133	92	26	0.291	0.702	0.199	0.792
Triglycerides, μmol/L	377[b]	375[ab]	12	0.897	429[a]	378[b]	16	0.044	0.067	0.092	0.096

[a-c] Different superscript letters within one row indicate $P < 0.05$ (Tukey test).
[1] NEFA = Non-esterified fatty acids.
[2] *P*-value from ANOVA analysis.
[3] *P*-value from Tukey slice test.

Table 5
Urea recycling and kidney function characteristics of dairy cows with low (LMU) and high milk urea concentration (HMU) under conditions of feeding 95% of the ad libitum intake of a normal (NP) and low (LP) crude protein diet. Data are given as least square means and standard error (SE).

Parameter[1]	NP				LP				*P*-value[3]		
	HMU	LMU	SE	*P*-value[4]	HMU	LMU	SE	*P*-value[4]	Diet	Group	Diet × group
^{13}C urea turnover, mg × kg^{-1} BW× d^{-1}	953^{a}	828^{ab}	50	0.097	749^{b}	691^{ab}	50	0.434	0.004	0.084	0.509
Urea pool size, mg/kg BW	174^{a}	139^{b}	9	0.014	121^{bc}	101^{ac}	9	0.144	<0.001	0.039	0.223
Urea-N pool size, g	52.9^{a}	43.5^{b}	2.7	0.020	36.8^{bc}	32.0^{ac}	3.2	0.307	<0.001	0.070	0.260
UER, g urea-N/d	291^{a}	261^{ab}	15	0.160	234^{b}	219^{ab}	19	0.591	0.008	0.215	0.630
K_{urea}, h^{-1}	0.224^{a}	0.262^{ab}	0.022	0.248	0.270^{b}	0.300^{ab}	0.020	0.294	0.010	0.210	0.798
Urea space, L	364	358	25	0.874	351	316	23	0.300	0.269	0.409	0.543
GER, g urea N/d	156	131	17	0.316	152	131	15	0.346	0.894	0.217	0.896
GER/UER, %	52.4^{cd}	48.1^{bd}	4.3	0.476	64.6^{ab}	59.4^{ac}	2.9	0.217	<0.001	0.273	0.889
UUN/UER, %	45.6^{ac}	50.3^{ab}	4.1	0.424	33.9^{bd}	39.3^{cd}	2.8	0.191	<0.001	0.230	0.915
Whole blood $^{13}CO_2/^{12}CO_2$, APE/d	0.09^{cd}	0.08^{bd}	0.01	0.568	0.12^{ab}	0.11^{ac}	0.01	0.600	0.002	0.506	0.849
UHR, mmol/30 min	50^{ab}	50^{b}	9	0.999	79^{ab}	66^{a}	15	0.914	0.008	0.937	0.906
Salivary urea, mmol/L	4.8^{a}	3.3^{bc}	0.5	0.050	3.3^{b}	2.5^{ac}	0.3	0.037	0.014	0.011	0.406
Salivary creatinine mmol/L	32.0	28.1	2.3	0.249	32.8	29.8	2.1	0.319	0.334	0.256	0.718
Rumen fluid NH_3, mmol/L	8.9^{a}	6.9^{ab}	0.8	0.103	5.8^{b}	5.4^{ab}	0.7	0.677	0.007	0.122	0.313
Fecal urease activity[2], mmol × kg^{-1} × min^{-1}	4.3	4.1	0.8	0.879	4.4	3.9	0.6	0.521	0.936	0.612	0.804
Urine excretion, L/d	14.9	15.1	0.9	0.908	14.0	14.6	1.0	0.699	0.411	0.730	0.799
CCR, L/min	0.88^{ab}	1.01^{ab}	0.09	0.306	0.85^{a}	1.06^{b}	0.07	0.049	0.921	0.100	0.471
UCR, L/min	0.62^{ab}	0.75^{a}	0.05	0.120	0.52^{ab}	0.61^{b}	0.04	0.189	0.020	0.057	0.621
UACR, L/min	0.86^{ab}	0.86^{b}	0.11	0.991	0.78^{b}	1.16^{a}	0.07	0.001	0.214	0.092	0.033
CREACR, L/min	0.20^{a}	0.32^{b}	0.02	<0.001	0.19^{ab}	0.24^{a}	0.02	0.099	0.011	0.004	0.018
RRR	0.32	0.26	0.06	0.499	0.37	0.42	0.06	0.505	0.098	0.961	0.354

$^{a-d}$Different letters within one row indicate $P < 0.05$ (Tukey test).
[1] CCR = renal creatinine clearance rate; CREACR = renal creatine clearance rate; GER = urea-N transfer to the gastrointestinal tract; K_{urea} = fractional disappearance rate of ^{13}C urea; RRR = renal urea reabsorption ratio; UACR = renal uric acid clearance rate; UCR = renal urea clearance rate; UER = urea-N entry rate; UHR = urea hydrolysis rate.
[2] Fecal urease activity was calculated to fecal DM excretion.
[3] *P*-value from ANOVA analysis.
[4] *P*-value from Tukey slice test.

2015) can be explained by the higher DMI and N supply supporting microbial protein synthesis. Nevertheless, other factors such as AD of accompanied feed components, rumen pH, the protein/energy ratio and stage of lactation determine NUE and animal performance as well (Hristov et al., 2004; Calsamiglia et al., 2010; Dijkstra et al., 2011). Comparing the phenotypes, HMU cows had higher milk fat percentage on both diets than LMU cows. As the mammary gland is a sink of blood TG, the higher milk fat secretion of HMU cows was likely due to higher plasma TG concentrations. Comparably, Hojman et al. (2004) and Godden et al. (2001) reported a positive association between MUN and milk fat content in Israeli and Canadian dairy herds, whereas in Western US herds, MUN and milk fat content were negatively associated (Johnson and Young, 2003). The discrepancy between studies can largely be explained by differences in ration composition and feeding regimes, with feeding the same TMR throughout lactation in Israel and performance-adjusted TMR feeding in the US. To achieve isocaloric conditions between our diets, predominantly starch was formulated to a higher portion in the LP diet. The metabolic HP, however, was smaller when animals were on the LP diet which was unexpected and contrast the findings by Hynes et al. (2016), who described no differences in HP between different CP feeding levels. The higher dietary starch and lower crude fiber content in the LP ration would reduce enteric methane emissions by shifting the ruminal VFA profile toward propionate formation (Bannink et al., 2010), but we did not observe differences in methane production between LP and NP diets. However, our results are in line with the statement of Niu et al. (2016), who described no effect of CP level on enteric methane emission for isocaloric diets balanced with fiber and starch. The increase in dietary CP content, however, increased total ammonia emissions from cow's excretions in average from 4.35 to 7.15 g/d. These numbers include ammonia originating from excreta and eructation, which are directly related to the CP level. In a study with defined manure slurries, ammonia emissions increased from 57 to 149 g of N/d per cow when the CP content of the ration increased from 15 to 21% of DM (Frank and Swensson, 2002; Burgos et al., 2007). Comparably, ammonia concentrations released from manure stored in ventilation chambers amounted to 8.8 ppm when cows were fed 17% CP, but only to 4.7 ppm with 13.5% CP feeding (Frank and Swensson, 2002; Burgos et al., 2007). The amount of ammonia released from excreta seems to be primarily dependent on the UUN level and less on fecal urease activity, as the latter was unaffected by the CP feeding level. There is a strong positive correlation ($R^2 = 0.85$) between ammonia emissions from manure and MUN (Frank and Swensson, 2002; Burgos et al., 2007), however, we did not find a significant group effect for ammonia emissions, likely because of the reasons described above.

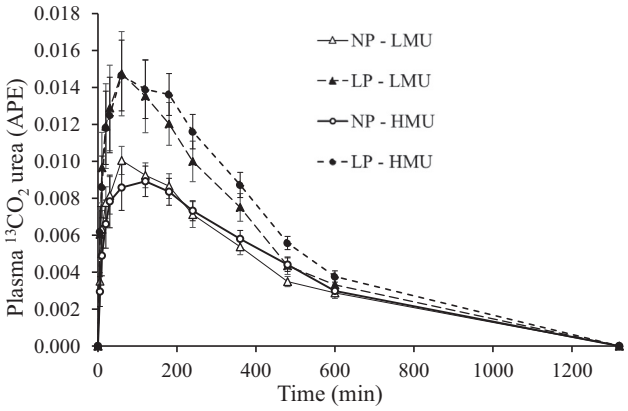

Fig. 3. Time course of $^{13}CO_2/^{12}CO_2$ enrichment in whole blood until 1320 min after i.v. ^{13}C urea tracer application in dairy cows grouped according to low (189 ± 12 mg/L; LMU) or high milk urea concentration (277 ± 9 mg/L; HMU) fed 15.8 ± 0.2% CP (normal protein; NP) or 13.7 ± 0.3% CP (low protein; LP). Data are shown as mean ± SEM. APE = atom percent excess.

4.2. Nitrogen balance

The higher N intake on the NP diet was accompanied with higher FN excretions. The increase in FN excretion with NP feeding can be attributed to increased excretion of undigested dietary CP, and a direct relationship between CP and FN output has been described previously (Kebreab et al., 2002; Broderick, 2003). Elevation in CP level usually causes an increase in

AD of N (Kebreab et al., 2002; Broderick, 2003; Hristov et al., 2004), improved N absorption and a decline in the percentage of FN relative to NI. We also found a higher AD of N accompanied by higher ruminal ammonia concentrations on the NP diet, but the latter was not effectively utilized for milk protein synthesis or N secretion with milk. Hristov et al. (2004) studied two rations with 15.5 and 18.3% CP levels and also found no effect of CP on the secretion of N with milk despite higher ruminal ammonia concentrations in cows fed the high-protein diet. The authors concluded that excess of RDP could not be efficiently utilized for microbial protein synthesis and was largely lost through urinary N excretion (Hristov et al., 2004). On the contrary, more microbial protein could have been synthesized with the higher CP level in the diet (Rojen et al., 2011; Spek et al., 2013a), which may have also contributed to the greater FN excretions, particularly in light of the comparable milk N output in our study. Accordingly, the increase in CP decreased the efficient conversion of dietary N into milk protein (Broderick, 2003; Hristov et al., 2004), which is comparable to our finding of a higher NUE when animals were fed the LP relative to NP diet. Urinary N excretions generally increase with increasing NI (Hristov et al., 2004). An almost zero N-balance was established for the LP diet, however, we did not account for dermal, hair, scuff losses from the animals. Although the sum of the latter is relatively small (Hristov et al., 2019), the "actual" N-balance might be even slightly lower than shown in Table 2. However, although cow groups did not differ in any parameter of N balance, they might be distinguished, besides MUN concentration, in the output of further distinct N-metabolites.

4.3. Excretions of N-metabolites via urine and renal functioning

In contrast to our hypothesis, HMU and LMU cows did not differ in UUN excretions; however, both phenotypes reduced UUN excretion when transferred from the NP to LP diet. This response can be explained, among others, by improvements in RRR with low-N supply (Rojen et al., 2011). The RRR increased from 42 to 56% and UCR by the kidney decreased from 35 to 25 L/h when arterial urea concentrations of Holstein cows decreased from ~7 to 3 mmol/L (Rojen et al., 2011). Furthermore, UCR decreased in average by 31% while RRR remained unchanged when cows were fed 11.6 vs. 15.3% CP in DM (Spek et al., 2013b), which is comparable to our result of a 21% lower UCR and increased RRR with LP feeding. However, UCR as well as CCR were 19 to 20% higher but PU concentrations lower in LMU cows, whereas again RRR was not affected by the phenotype. This finding is surprising as the reduction in PU concentration was described directly associated with the reduction in UCR to allow for greater urea N recycling (Rojen et al., 2011). Thus, the lower PU concentrations are unlikely the reason for the higher UCR in LMU cows. Rather, the higher UCR and CCR, the latter considered an estimate of the glomerular filtration rate (Spek et al., 2013a), have resulted in the lower urea pool size, lower PU concentrations and diminished urea turnover in LMU cows. This assumption is further supported by the negative correlation between CCR and UCR with milk urea concentration. The mechanism for the higher UCR despite lower PU concentrations is unknown but likely involves different urea transport processes and kinetics. This could be accomplished by the involvement of various isoforms of the vasopressin-regulated urea transporter in the kidney medulla (UT-A1, A2), the erythrocyte urea transporter (UT-B) (Sands, 1999), several aquaporin proteins such as the vasopressin-dependent aquaporin 2 (AQP 2), expressed along the collecting duct, and the vasopressin-independent aquaporin 1 (AQP 1), located in the proximal nephron (Elfers et al., 2014). Also, the involvement of osmolytic factors regulating urea excretion, i.e. uric acid or creatine, cannot be excluded (see below). Another reason for the lower PU concentration and urea turnover may be a reduced N-flux through the urea cycle in the liver and kidney. This assumption is supported by lower plasma Cit concentrations in LMU cows, however, other metabolites of the urea cycle such as Orn and Arg did not differ between phenotypes. The major source of uric acid is microbial nucleic acids, which are catabolized via hypoxanthine and xanthine in the small intestine. These purine derivatives are absorbed and primarily excreted via urine, and therefore considered as markers of microbial N flow to the duodenum (Broderick, 2003; Tas and Susenbeth, 2007; Dijkstra et al., 2013). We found urinary uric acid excretion increased when LMU but not HMU cows were transferred from the NP to LP ration, suggesting a higher microbial N flow in LMU cows with LP compared to NP feeding. Moreover, the increase in urinary uric acid excretion was accompanied by an increase in UACR when the CP level was reduced for LMU cows. On the LP diet, LMU cows had even lower plasma uric acid concentrations, suggesting increased activation of renal uric acid transporters. Urinary uric acid excretion seems not to be influenced by urinary or plasma urea concentrations, as an earlier finding demonstrated no alterations in urinary uric acid excretion when dairy cows were ruminally infused with 0, 4.1 and 8.5 g urea per kg DMI (Rojen et al., 2011). On the other hand, Giesecke et al. (1994) reported that the urinary uric acid excretion rate varies between 12.7 and 35.2 mmol/d when cows were fed rations containing a CP level of 13.8 and 15% of DM, however, it remained unclear if the variation in urinary uric acid excretion was due to dietary or inter-individual reasons. However, as the N_2O emission factor for uric acid is the highest of all non-UUN compounds (Gardiner et al., 2018), it seems that the urine from HMU compared to LMU cows fed the LP diet has a less intense environmental nitrogen footprint.

If not excreted with urine or secreted with milk, uric acid is degraded by uricase in the liver yielding allantoin, but urinary allantoin excretion was not different between groups. It is of interest to further investigate if osmolytic properties of uric acid or other metabolites such as creatine are responsible for the different UCR, or if the osmolytic characteristics of urea affect UACR and CREACR in these cows. Both, CREACR and total urinary creatine excretion also decreased with decreasing CP level and were higher in LMU than HMU cows. Creatine is primarily produced in kidney, liver and pancreas, but predominantly stored as phospho-creatine in skeletal muscle. Creatine biosynthesis is accomplished from guanidinoacetate, which in turn is synthesized from Gly and Arg via the Arg:Gly amidinotransferase (AGAT). Interestingly, the concentration of Gly and its precursor Ser increased in plasma when LMU but not HMU cows were fed the LP compared to NP diet, however, the creatine metabolism in these cows needs to be studied in future. Besides Gly, Arg is required for creatine synthesis, but plasma Arg concentrations were not different between cow groups. Arg is converted in the urea cycle by arginase 1 (ARG1), resulting in the formation of urea and Orn (Nelson and Cox, 2013). Thereby, creatine biosynthesis competes with urea synthesis for Arg utilization, but again, this aspect should be subject of future studies. The different fates of ammonia N, either directed toward creatine or urea synthesis, and their excretions via urine has consequences for the ammonia and nitrous oxide (N_2O) emissions from manure or urine patches on the soil of pasture. Creatine decomposes more slowly than urea in soil, and ammonia volatilization from urinary N-metabolites decrease in the order urea > allantoin > creatinine > creatine > hippuric acid (Whitehead et al., 1989; Dijkstra et al., 2013). Also, the N_2O emission factor for creatine is the lowest of all non-UUN compounds (Gardiner et al., 2018), suggesting slower and less intense nitrogen emissions from the urine of HMU relative to LMU cows. The inter-individual variation in creatine excretions is largely unknown, but this trait might be considered in further to ameliorate the environmental N-footprint from ruminants.

4.4. Plasma-milk-urinary urea relationships and uric acid secretion with milk

The MUN excretion is, in line with previous studies (Burgos et al., 2007; Niu et al., 2016), positively related with CP level and PU concentration. Also, Gustafsson and Palmquist (1993) described serum and MUN concentrations to be strongly correlated, which is reasoned by the bi-directional urea transfer between plasma and milk (Spek et al., 2016). Moreover, blood urea concentrations were proposed to quantify

N excretion in various species (Kohn et al., 2005), whereas based on a meta-analysis performed by Spek et al. (2013c), it was concluded that UUN excretion can be better predicted by the combination of MUN secretion and CP level. Our findings on a positive correlation between UUN and MUN excretions for the LP but negative correlation for the NP diet, with R^2 values smaller than those reported earlier ($R^2 > 0.9$ in Burgos et al. (2007)), and the group difference in the UUN/MUN excretion ratio underline that MUN secretion cannot accurately predict UUN excretion for all cows and diets. These results further indicate that genetic selection for MUN will not reduce total urinary N excretion, as it was proposed recently (Beatson et al., 2019). Milk uric acid secretion increased by 15% with the lower CP level in our study, and therefore cannot be used as marker for intestinal microbial N flow (Tas and Susenbeth, 2007). Besides, the single nucleotide polymorphism in the ATP-binding cassette transporter G2/breast cancer resistance protein (ABCG2 Y581S) was described to increase uric acid transport from plasma into milk (Otero et al., 2015), but if this polymorphism accounts for the observed diet x group interaction for uric acid secretion needs to be determined in future studies.

4.5. Urea entry rate, transfer to the gastrointestinal tract and into urine

According to Lapierre and Lobley (2001) on average one-third of hepatic urea N flux is eliminated with urine, while two-third is transferred into the gastrointestinal tract for anabolic purposes. However, Spek et al. (2013a) reported a higher portion of urea transferred to the gastrointestinal tract, ranging from 84.7 to 74.1% at CP levels of 11.6 and 15.4% of DM, respectively. In our study, we found GER in a range between 48.1 and 64.6%, which is more comparable to (Lapierre and Lobley, 2001; Spek et al., 2013a). This discrepancy with the latter study can be likely attributed to our two-exponential model illustrated in Fig. 1, revealing a better fit compared to the one-exponential regression used by Spek et al. (2013a). However, alike as reported earlier (Spek et al., 2013a), UER decreased but the GER/UER ratio increased with lowered CP level, indicating an improved N use efficiency on the LP diet. Furthermore, reduced salivary and plasma urea concentrations reflect the decrease in UER with reduction of CP feeding. The proportional increase in GER with LP feeding was not accompanied by an increase in absolute GER, as shown previously (Calsamiglia et al., 2010; Rojen et al., 2011; Mutsvangwa et al., 2016). Nevertheless, UHR was higher when cows were fed the LP ration, indicating that urease activity is not solely substrate-regulated but potentially inhibited. Numerous plant compounds are known to inhibit urease (Amtul et al., 2002), but if those were more abundant in either of the diets could not be analyzed in the present study. The greater UHR, however, did not result in higher ammonia concentrations in the rumen. Rather, ruminal ammonia concentrations were lower on the LP diet. This is because only 20% of ruminal ammonia originates from endogenous urea while the majority (80%) is produced from feed (Lapierre et al., 2005). Another reason is that on a reduced dietary CP level, ammonia is more rapidly utilized by rumen microbes and less concentrated in rumen fluid. Lower ruminal ammonia concentrations facilitate the urea transport across the ruminal wall (Lu et al., 2014; Patra and Aschenbach, 2018), and thus, more blood urea N is incorporated into bacterial protein with decreased NI (Bunting et al., 1987). Collectively, these data show that microbial protein synthesis is more dependent on recycled urea N when dietary NI decreases. Furthermore, microbial protein synthesis is limited when LMU cows are transferred from the NP to LP diet, as indicated by the increase in plasma α-aminobutyric acid, methylhistidine and Gln concentrations, suggesting increased endogenous protein breakdown and transfer of extrahepatic N to the liver. On the other hand, plasma NEFA concentrations were not affected by the diet, indicating no body fat mobilization with reduced CP feeding.

Although the urea turnover (UER normalized to BW) tended to be lower for LMU than HMU cows, UER was comparable between groups. Furthermore, the absolute and relative urea N transfer into the rumen-intestinal tract and the relative urea N excretion with urine were not different between cow groups. However, LMU cows had a lower urea pool size but higher UCR, indicating that cows with lower milk urea secretion have a better renal performance for urea elimination resulting in a lower urea body pool without affecting GER.

5. Conclusion

Reducing the dietary CP level from roughly 16 to 14% on the dry matter basis is most effective in reducing urinary nitrogen excretions, ammonia emissions from excreta, and in tendency fecal nitrogen excretions, without compromising milk yield of non-pregnant, late lactating Holstein cows. Whether the reduced dietary CP level actually affects endogenous protein breakdown should be more intensively investigated in follow-up studies. Dairy cows with low milk urea secretion did not excrete less urinary urea and total nitrogen than cows with high milk urea secretion as we hypothesized. Rather, dairy cows with higher milk urea secretion tend to synthesize more urea and have worse renal performance in respect to the urea clearance rate resulting in a greater urea pool size. The latter allows for more urea transfer to the mammary gland without affecting total urinary urea excretion. And although the amount of non-urea urine nitrogen excretion was not different between cow groups, animals with high milk urea concentrations excrete less urinary creatine. If urine from the latter cows has therefore a less intense environmental nitrogen footprint requires experimental confirmation in near future.

CRediT authorship contribution statement

C.B.M.M. conducted the animal experiment, provided medical care, performed statistical analysis. C.B.M.M. and B.K. wrote the manuscript. S.G. performed mass spectrometric and HPLC analyses, and calculated pool, turnover and excretion rates. M.D. performed measurements with respiration chambers. A.Z. measured gross energy in a bomb calorimeter. A.T. developed the statistical models and performed statistical analysis. K.W. and BK jointly designed and supervised the animal experiment. They contributed to the interpretation of the data and to the writing of the manuscript. All authors approved the final manuscript as submitted.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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