Variation of milk urea in dairy cattle

A study on factors that affect the relationship between urea concentration in milk and urea excretion in urine

Jan Wouter Spek

Thesis committee

Promotor
Prof. Dr W.H. Hendriks
Professor of Animal Nutrition
Wageningen University

Co-promotors
Dr J. Dijkstra
Associate professor, Animal Nutrition Group
Wageningen university

Dr A. Bannink
Senior researcher, Wageningen UR Livestock Research, Lelystad

Other members
Dr S. de Campeneere, ILVO, Belgium
Prof. Dr O. Oenema, Alterra Wageningen UR
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Dr M.R. Weisbjerg, Aarhus University, Denmark

The research was conducted under the auspices of the Graduate School of Wageningen Institute of Animal Sciences (WIAS)

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Thesis

submitted in fulfilment of the requirements for the degree of doctor
at Wageningen University
by the authority of the Rector Magnificus
Prof. Dr M.J. Kropff,
in the presence of the
Thesis Committee appointed by the Academic Board
to be defended in public
on Monday 30 September 2013
at 4 p.m. in the Aula.

Jan Wouter Spek
Variation of milk urea in dairy cattle. A study on factors that affect the relationship between urea concentration in milk and urea excretion in urine, 162 pages

PhD thesis, Wageningen University, Wageningen, NL (2013)
With references, with summaries in Dutch and English

ISBN 978-94-6173-664-2

Abstract

The aim of this thesis was to increase the applicability of milk urea nitrogen concentration (MUN) as a predictor of urinary urea nitrogen excretion (UUN) by identifying and quantifying factors that can explain variation in MUN that is not related to UUN. A literature study was conducted in order to identify these factors that affect the relationship between MUN and UUN. In this literature review a number of factors were established that affect the relationship between MUN and urinary N-excretion (UN) or UUN, such as dietary crude protein content (CP), intake of dietary salt and water, body weight, diurnal variation in plasma urea nitrogen concentration (PUN), exchange of urea between blood and milk, and heritability of MUN. Results of a quantitative meta-analysis where the effect of various physiological and dietary factors on the relationship between MUN and UN or UUN were studied confirmed the fact that CP affects the relationship between MUN and UUN and showed that by using information on MUN and CP more variation in UUN could be explained compared to using information on either MUN or CP alone. One of the factors established in the literature review that can affect the relationship between MUN and UUN is dietary salt content or drink water intake. In order to quantify the effect of dietary salt on MUN and UUN an experiment was carried out that investigated the effect of four dietary levels of sodium chloride (NaCl) on urea levels in blood plasma and milk and on UN and UUN. The results from this trial clearly showed a negative relationship between dietary NaCl content and MUN whereas UUN was not affected by NaCl intake and UN was slightly increased by increasing NaCl intake levels. The question arose whether the effect of dietary salt on MUN would be similar at high and low dietary protein levels as the renal mechanism of excretion and reabsorption of urea is affected by both dietary protein and salt intake. Therefore, the interaction between dietary salt and protein on UUN was tested in an experiment with two CP levels and two dietary NaCl levels. No interaction between dietary NaCl and CP on MUN was observed. However, the relationship between MUN and UUN was altered by the effect of salt intake. The literature review showed that diurnal variation in PUN and MUN can be substantial, and that this variation depends on factors such as time and frequency of feeding and milking. Insight in the dynamics of urea transport between blood of milk is important in order to model and predict variation in MUN over time under various feeding and milking regimes. To obtain quantitative insight in urea fluxes between blood and milk two experiments were conducted in which urea transport from blood to milk and vice versa was investigated by means of pulse dose injections of labeled [$^{15}N^{15}N$]urea in milk cisterns at various time intervals before milking. The results showed a rapid distribution of injected labeled urea throughout the milk in the mammary gland and substantial urea transport from milk to blood.

It is concluded that various factors that are discussed in this thesis contribute to variation in MUN that is not related to UUN. Taking these factors into account increases the applicability of MUN as a predictor of UUN.

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Chapter 1

General introduction

Introduction

After the Second World War agricultural production in Europe was stimulated by means of research, extension, education, and intervention prices in order to prevent food shortages experienced during the Second World War. This stimulation resulted in, amongst others, an increase in the number of dairy cows. For example, in the Netherlands the number of dairy cows increased from 1.5 to 2.4 million cows from 1950 up to 1980 although the area of land used for dairy farming did not increase during this period (CBS-Statline, 2013). This increase in the number of dairy cows per unit of land became possible by increased inputs of the minerals nitrogen (N), phosphorus (P), and potassium (originating from concentrates and artificial fertilizer) per unit of agricultural land. For example, fertilizer N-input in Dutch agriculture more than doubled from 1950 (156 million ton) to 1980 (485 million ton) (Oenema and Berentsen, 2005) with an average 63% of the agricultural land being used for dairy farming during that period. Although this resulted in an increased milk production per unit of land used for dairy farming it also resulted in large losses of N per ha of land. For example, in 1986, the earliest year from which N-input and output information per ha can be obtained from the CBS-Statline, an average N-loss of 265 kg N per year per agricultural ha of land was calculated in the Netherlands. These large N-losses to the environment had negative consequences for the balance and heterogeneity of ecosystems and the quality of surface and ground water.

Because of these negative side effects, and also because over-production resulted in large surpluses of milk and butter, Dutch policies were directed towards halting the increase in milk production and reducing N-losses per ha. In 1984 in the European Community, the milk quota system was introduced under the Dairy Produce Quota Regulations in order to restrain rising milk production. Furthermore, in the Netherlands, in 1987 manure production quota per farm and limits for manure application to land based on P were implemented. In 1998 another system was introduced called MINAS (Mineral Accounting System) that set limits to the losses of P and N per ha, accounting for the use of artificial nitrogen fertilizers as well.

Furthermore, because of 1) the negative effects of ammonia (NH_3) emission on the environment such as acid rain formation, eutrophication of soils and surface waters, and fine particulate matter formation (Fangmeier et al., 1994), and 2) the large role of agriculture, especially from cattle husbandry, in contributing to NH_3 emission (Pain et al., 1998), policy measures have been taken in the Netherlands to reduce NH_3 emission in agriculture. These measures consisted of; covering manure storages, low emission animal housing systems, limiting the period of the year when manure is allowed to be applied to the land, and making it compulsory to inject manure into the soil. Because of the positive relation established between ammonia emission and urea concentration in milk (Van Duinkerken et al., 2005) in 2002 an agreement was made between the Dutch government and the dairy farmers to reduce the milk urea concentration to 20 mg urea/dL. In return the Dutch farmers would not be obliged to invest in low emission housing systems.

However, because of failure to comply with the European Nitrate Directive from 1991 that limits the maximum allowable nitrate level in ground water to 50 mg/L, a new legislation policy was introduced in 2006 (till present) in the Netherlands based on the requirement of the crop. Depending on the area of land, the type of crop (grass or maize), and availability of N and P in the soil, a dairy farmer is allowed to apply a certain quantity of N and P to the land.
The measures for the dairy sector (described above) and measures taken for agriculture in general have had a desired effect on nitrate levels in surface and ground water levels and ammonia emission in such a way that its goals, the European Nitrate Directive from 1991 (Fig. 1) and the National Emission Ceilings Directive from 2001 (Fig. 2), have been largely reached.

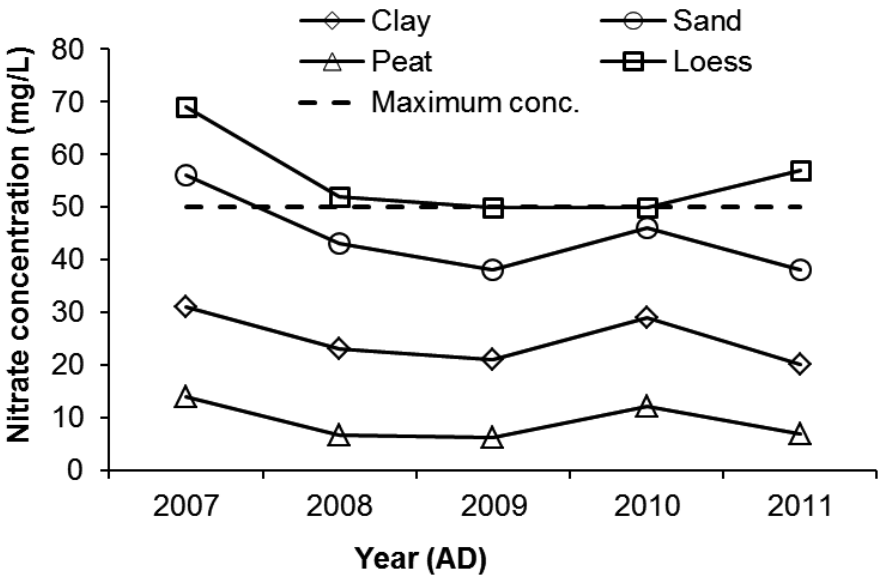

Figure 1. Average nitrate concentrations in water coming directly from the grass root zone from grass land areas on clay, peat, sand, and loesss soil during the years 2007 – 2011 for 290 grass land farms in the Netherlands. The maximum allowable nitrate concentration of 50 mg/L. Source RIVM (2012).

Although on average the goals with regard to the reduction of nitrate levels and NH_3 emission have been largely reached, for a number of dairy farms (39% of farms located on sand soils and 44% of farms located on loess soils (RIVM, 2012)) nitrate levels were higher than the maximum tolerable level of 50 mg/L. As well, the agreement of the dairy sector with the government to reduce milk urea levels to 20 mg urea/dL has not been fulfilled (on average 22 mg/dl during the years 2009 to 2011 (Boerderij, 3 April 2012).

The concentration of milk urea is not only used as an instrument to reduce ammonia emission, it is also used for estimating the quantity of N excreted by dairy cows. For dairy farmers, the room for importing N via artificial or organic manure is determined by the difference between the quantity of N that is allowed to be used and the quantity of nitrogen in animal manure. In the default situation the quantity of N in manure (feces plus urine) from dairy

cattle is estimated based on the urea concentration in the milk and the average quantity of milk produced per cow per year. In this estimation the quantity of N excreted in manure is positively related to the concentration of milk urea nitrogen (MUN; mg N/dL) and the average quantity of milk produced per cow per year.

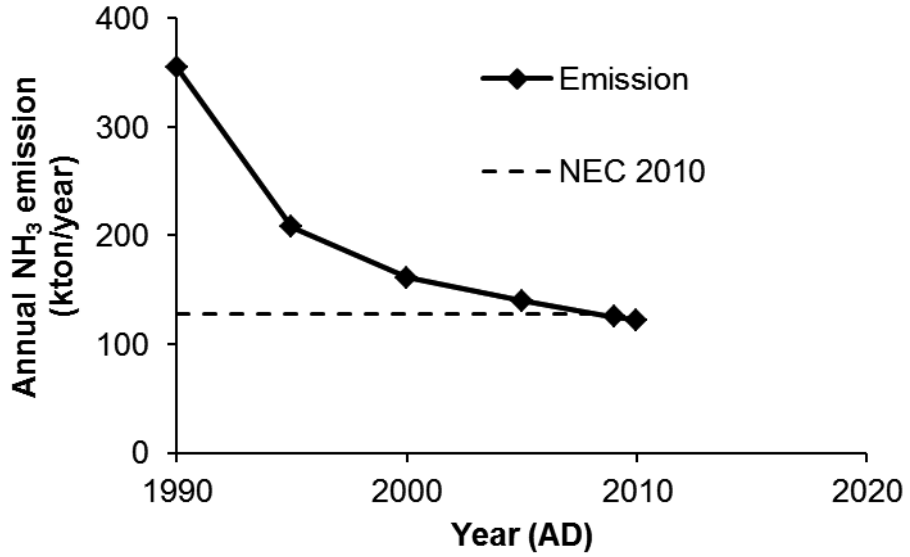

Figure 2. Annual ammonia (NH_3) emission in the Netherlands from 1990 – 2010. The National Emission Ceiling (NEC) was set at 128 kton/year for 2010. Source CBS-Statline (2013).

Indeed, there is a considerable body of evidence for a positive and strong relationship between MUN and urinary nitrogen excretion (UN) (Ciszuk and Gebregziabher, 1994; Jonker et al., 1998; Kauffman and St-Pierre, 2001; Bannink and Hindle, 2003; Broderick, 2003; Nousiainen et al., 2004; Burgos et al., 2007). Although all studies find a positive relationship between MUN and UN, studies sometimes differ substantially in estimated slope and intercepts. For example, using the regression equations from the studies of Broderick (2003) and Nousianen et al. (2004), a MUN of 5 mg N/dL results in predicted UN of 168 and 108 g/d, respectively, and a MUN of 15 mg N/dl in UN of 280 and 235 g/d, respectively. Clearly, there is a considerable between-experiment variation. Data from Dutch N-balance trials evaluated by Bannink and Hindle (2003) show that a significant proportion of variation (28%) of UN of individual animals remains unexplained by MUN (MUN ranged from 5 to 30 mg N/dl) and that this was associated with a root mean square error (RMSE) of 47.5 g/d which is too large for UN prediction purposes (Fig. 3). However, upon using a subsample of this dataset in a limited MUN range of 5 - 15 mg N/dL, which is of more practical relevance because this is the typical range of MUN values established on commercial farms, the relationship between UN and MUN is poor (R^2 = 0.23 and RMSE = 44.1). This poor relationship between UN and MUN within the typical range of MUN values experienced on commercial farms raises the question if milk urea is an appropriate instrument to reduce ammonia emission and estimate N-excretion.

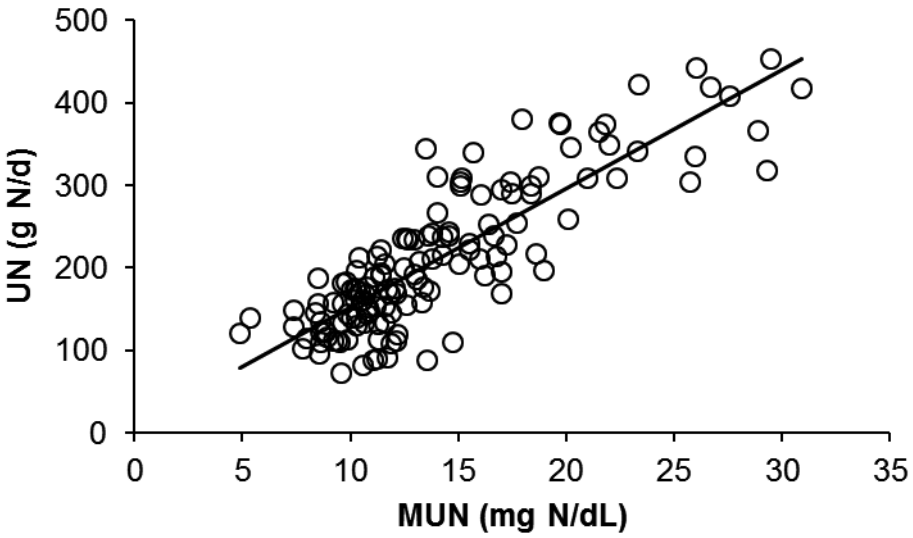

Figure 3. Linear regression of urinary nitrogen excretion (UN; g N/d) against milk urea nitrogen concentration (MUN; mg N/dL) for observations from individual animals from 9 experiments evaluated by Bannink and Hindle (2003). The result of regression is indicated by solid line described by UN (g N/d) = 8.1±11.57 + 14.41±0.771 × MUN (mg N/dL), R^2=0.72, RMSE=47.5 and CV of 22.5%.

An alternative option is available for dairy farmers in the Netherlands to quantify manure N-excretion. In this alternative, N-excretion in manure is estimated based on the net energy and N-content of the feedstuffs fed, the quantities of feedstuffs fed, the calculated energy requirement of the animals and the quantity of N retained in milk and tissue. However, this way of estimating N-excretion in manure is carried out once a year and is therefore unsuitable as a management tool for minimization of N-losses in manure on a daily basis.

The parameter MUN, on the other hand, can provide information on N-excretion on an (almost) daily basis (Jenkins et al., 1999) and thereby fulfills one of the requirements of an on-farm management tool to minimize N-excretion in manure. The second requirement for MUN in order to serve as an on-farm management tool is accuracy and precision. At the moment this accuracy and precision is lacking as can be judged from the poor relationship between UN and MUN with the typical range of MUN values experienced in practice. A wrong interpretation of MUN may result in under feeding of protein, resulting in a reduced milk production, or over feeding, resulting in unnecessary high feed costs (as dietary protein is an expensive nutrient), and increased losses of N to the environment with its negative consequences.

If the accuracy and precision of MUN as an estimator of urinary urea N excretion (UUN) can be increased this would greatly enhance its applicability in reducing ammonia emission and it would enable the dairy farmer to take informed decisions on an almost daily basis with respect to optimization of N-utilization and minimization of N-losses via urea.

Aim of this Thesis

In order to increase the applicability of MUN the general aim of this thesis was to identify and quantify those factors that affect the relationship between MUN and UUN and that can explain that part of the variation in MUN that is not related to UUN. Furthermore, the findings from this thesis will be used in the development of a mathematical model that aims to enable a better interpretation of MUN under various circumstances.

Thesis Outline

This thesis describes the results of seven studies consisting of a literature review, a meta-analysis study, four trials and a general discussion. In **Chapter 2** literature is reviewed on factors that affect MUN, the relationship between MUN and UN, or the relationship between MUN and UUN. **Chapter 3** is a quantitative meta-analysis on prediction of UN and UUN by means of various animal and dietary related factors for Northwestern Europe and North America. From literature it appeared that intake of salt or water affects MUN and the relationship between MUN and UN or UUN, but an estimate of the size of this effect remained unclear. Therefore, a trial was conducted and described in **Chapter 4** that studied the effect of dietary NaCl intake on the relationship between MUN and UUN or UN. Because the effect of dietary salt intake on the relationship between MUN and UUN might dependent on the level of protein intake, another trial was conducted on the interaction between dietary content of protein and NaCl on the relationship between MUN and UUN (**Chapter 5**). Further, there might be a diurnal variation in plasma urea and MUN that is affected by factors such as the frequency of feeding and milking. After a meal the concentration of ammonia in the rumen is likely to increase due to rumen microbial degradation of dietary protein. Rumen ammonia is absorbed from the rumen to blood and subsequently converted into urea by the liver. Therefore, after a meal, an increase in the level of urea in blood is expected, followed by an increase in MUN due to diffusion of urea between blood and milk. Quantitative insight in the diffusion between milk and blood is needed to understand how frequency of feeding and milking and of blood urea dynamics affect MUN. However, quantitative insight in diffusion of urea between blood and milk is lacking. Therefore, a pilot study was carried out that studied urea transfer between milk and blood. The methodology and results of this pilot study are described in **Chapter 6**. On the basis of this pilot study a main experiment was carried out that studied urea transport from milk to blood and within the mammary gland, and is described in **Chapter 7**. In the general discussion in **Chapter 8** the factors that were observed in previous chapters to affect the relationship between MUN and UUN were discussed and, through modeling exercises, the effect of these factors, mediated through renal characteristics such as the glomerular filtration rate and renal recycling of urea excreted in the glomerular filtrate, on the relationship between MUN and UUN were simulated.

Chapter 2

A review of factors influencing milk urea concentration and its relationship with urinary urea excretion in lactating dairy cattle

J.W. Spek[1,2], J. Dijkstra[1], G. van Duinkerken[2], and A. Bannink[2]

[1]Animal Nutrition Group, Wageningen University, PO Box 338, 6700 AH, Wageningen, The Netherlands
[2]Department of Animal Nutrition, Wageningen UR Livestock Research, PO Box 65, 8200 AB, Lelystad, The Netherlands

Journal of Agricultural Science (2013) 151, 407-423

Abstract

Milk urea nitrogen (MUN) concentration in dairy cows may serve as an on-farm indicator to guide nutritional strategies and to help reduce emissions of nitrogen (N) to the environment. Excretion of urinary urea nitrogen (UUN) is positively related to MUN but the relationship is highly variable. The accuracy of MUN as a predictor of UUN may improve when various factors that affect this relationship can be taken into account. The current review discusses the impact of a number of UUN:MUN ratio influencing factors related to: physiological mechanisms in the dairy cow, farm management, differences between individual cows, nutrition, and analysis methods for MUN. Factors related to variation in water intake, urine production, dietary protein level, body weight, and time and frequency of feeding and milking are shown to affect MUN and its relationship with UUN. In addition, a number of factors are discussed that are likely to affect this relationship such as biological rhythm, renal reabsorption of urea during periods of protein deficiency, and breeding value for MUN. Accounting for these above-mentioned factors in the relationship between MUN and UUN might improve substantially the applicability and accuracy of MUN as a predictor of protein utilization efficiency and UUN.

Introduction

Reducing nitrogen (N) excretion by dairy cattle is desirable due to global concerns about the agricultural contribution to environmental pollution by N, particularly as ammonia volatilization into the atmosphere and nitrate leaching into surface and groundwater (Draaijers et al., 1989; Howarth et al., 1996). The primary source of ammonia-N from manure is urinary urea-N (UUN; g N/d), which is hydrolyzed to ammonia and carbon dioxide by the activity of microbial urease present in feces. Urea is formed mainly in the liver as a means of detoxification of ammonia present in the systemic circulation, is transported in blood plasma (plasma urea N; PUN; mg N/dL), and subsequently diffuses or is transported to other fluid pools in the body such as milk in the udder and liquid in the rumen. Urea is excreted with the urine and most urea is eliminated from blood with urine excretion. Urea accounts for 0.50 – 0.90 of the N in cattle urine (Bristow et al., 1992). An increase in N-intake leads to a more pronounced increase in urinary total N-excretion (UN; g N/d) than in fecal N excretion (Jonker et al., 1998; Kebreab et al., 2002) and generally results in an elevated concentration of milk urea-N (MUN; mg N/dL).

Hence, clear positive correlations have been shown between MUN and UN (Ciszuk and Gebregziabher, 1994) and between MUN and UUN (Burgos et al., 2007), and significant relationships have also been shown between ammonia emission and UUN (Burgos et al., 2010) and between ammonia emission and MUN (Van Duinkerken et al., 2005). This has led to the development of several models predicting UN from MUN alone (Jonker et al., 1998; Broderick, 2003), from MUN and daily milk production (Bannink and Hindle, 2003; Nousiainen et al., 2004), from MUN and bodyweight (Kauffman and St-Pierre, 2001; Kohn et al., 2002) or from MUN, daily milk production and milk protein yield (Bannink and Hindle, 2003).

Although correlation coefficients appear reasonably high, estimates of UN excretion using various equations based on MUN differ considerably (Fig. 1). For example, using the regression equations from the studies of Broderick (2003) and Bannink and Hindle (2003), a MUN value of 10.0 mg N/dL results in a predicted UN of 223 and 128 g N/d, respectively. Clearly, there is considerable between-experiment variation. Data from Dutch N-balance trials (Bannink and Hindle, 2003) show that a significant proportion of variation (c. 0.72) of UN is explained by MUN (MUN ranged from 5.0 – 30.0 mg N/dL). However, upon using a subsample of this dataset in a limited MUN range of 5.0 – 15.0 mg N/dL, often observed in practice, prediction accuracy of UN by MUN was low ($R^2 = 0.23$). Apparently some key factors are not accounted for in these equations, leading to substantial differences in predicted UN.

Under a wide range of conditions, dietary protein concentration explains most variation in MUN (Broderick and Clayton, 1997; Nousiainen et al., 2004) and UN (Nousiainen et al., 2004), and the effect of the amount and type of dietary protein on MUN and N-excretion has been studied frequently (e.g. Oltner et al., 1985; Broderick and Clayton, 1997; Broderick, 2003; Nousiainen et al., 2004). However, besides dietary protein, several factors affect MUN and possibly the relationship between MUN and UN or UUN (Table 1). Taking these factors into

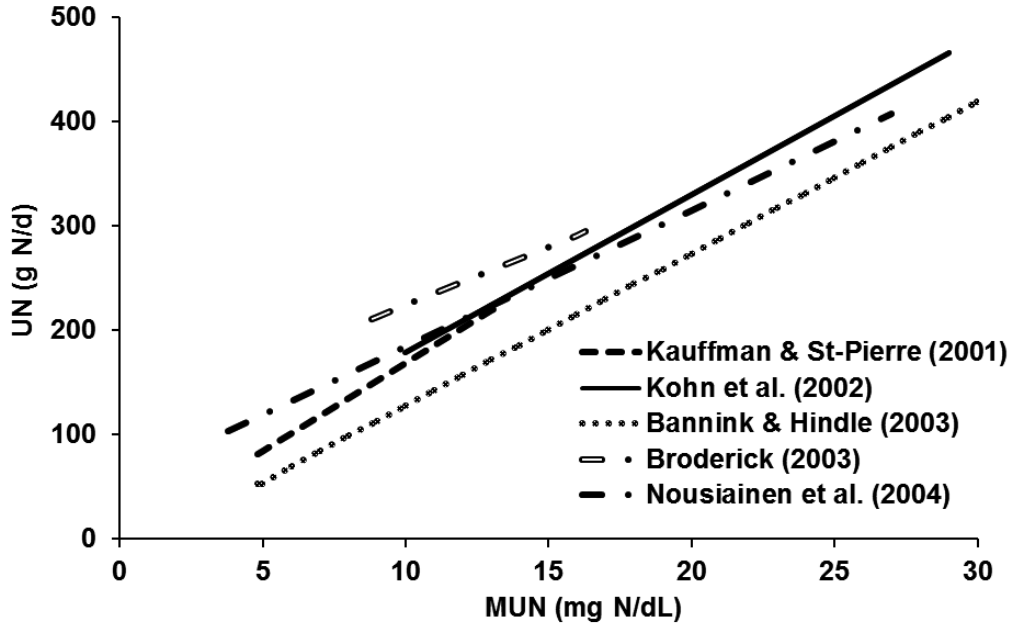

Figure 1. Effect of milk urea nitrogen concentration (MUN; mg N/dL) on predicted daily urinary N-excretion (UN; g/d) of a cow having a body weight (BW; kg) of 650 kg and producing 30 kg of milk/d (DMP) based on equations of Kohn et al. (2002); UN = 15.1 × MUN + 27.8, Kauffman and St-Pierre (2001); UN = 0.0259 × BW × MUN, Broderick (2003); UN = 11.2 × MUN + 111.6, Bannink and Hindle (2003); UN = 14.5 × MUN – 4.79 × DMP + 126.3, and Nousiainen et al. (2004); UN = 13.1 × MUN + 6.00 × DMP. The MUN range of the model prediction curves correspond with the range of observed MUN values of the datasets on which the model equations are based on

account may improve the accuracy of prediction of UN or UUN from MUN in combination with these factors.

In the current review, the effect of a number of factors are summarized and evaluated for their possible impact on the relationship between MUN and UUN. More literature is available with respect to the relationship between MUN and UN than between MUN and UUN. Because the concentration of urea-N and N in urine are closely associated (Burgos et al., 2005), and because of the strong relationship between UN and UUN that was established in the current study (Fig. 2), research evidence on the relationship between MUN and UN was taken into consideration as well.

Figure 3 provides a simplified representation of flows that affect PUN, MUN, UN, UUN, and the relationship between MUN and UUN or UN. These relationships can be expressed as UUN:MUN and UN:MUN ratios, respectively. The N-flows presented in Fig. 3 are dynamic flows that depend on a combination of factors such as management, nutrition, and cow factors. The first section describes some physiological factors that are likely to affect the relationship between MUN and UUN. Making use of the physiological principles described in the first section, the following sections of the current review focus on factors related to management, differences between cows and nutrition.

Table 1. Overview of differences in ratios between urine nitrogen excretion (UN; g N/d) and milk urea nitrogen (MUN; mg N/dL), between urinary urea nitrogen excretion (UUN; g N/d) and MUN, between UN and plasma urea nitrogen (PUN; mg N/dL), and between UUN and PUN in bovines at treatment contrasts from various studies.

			Ratio values	
Reference	**Contrast**	**Type of ratio**	**Treatment A**	**Treatment B**
Kaufman and St-Pierre (2001)	Holstein vs. Jersey	UN:MUN	11.8 for Jersey	17.6 for Holstein
Broderick and Clayton (1997)	Daytime vs. Nighttime	UN:MUN	4.6 at daytime	7.8 at night time
Hristov et al. (2005)	NDF vs. starch rich diet	UN:MUN	15.1 for NDF rich diet	16.4 for starch rich diet
Beckman and Weiss (2005)	NDF:Starch ratio	UN:MUN	12.1 for NDF:starch ratio of 1.27	12.6 for NDF:starch ratio of 0.74
Broderick (2003)	high NDF (360 g kg/DM) vs. low NDF (280 g kg/DM) diet	UN:MUN	15.0 for high NDF diet	15.7 for low NDF diet
		UUN:MUN	13.6 for high NDF diet	13.7 for low NDF diet
De Campeneere et al. (2006)	Grass silage vs. maize silage diet	UN:MUN	11.1 for maize silage diet	13.9 for grass silage diet
Olmos Colmenero and Broderick (2006)	Low protein (156 g kg/DM) vs. high protein (176 g kg/DM) diet	UN:MUN	14.6 for low protein diet	16.5 for high protein diet
		UUN:MUN	8.2 for low protein diet	13.3 for high protein diet

Table 1 Continued

Broderick (2003)	Low protein (151 g kg/DM) vs. high protein (184 g kg/DM) diet	UN:MUN	15.2 for low protein diet	14.8 for high protein diet
		UUN:MUN	12.9 for low protein diet	13.6 for high protein diet
Borucki Castro et al. (2008)	Low protein (162 g kg/DM) vs. high protein (201 g kg/DM) diet	UN:MUN	13.7 for low protein diet	15.4 for high protein diet
		UUN:MUN	10.2 for low protein diet	13.5 for high protein diet
Haig et al. (2002)	Low NPN concentration of dietary CP (33 g CP/kg DM) vs. high NPN concentration of dietary CP (117 g CP/kg DM). NPN increase from urea	UN:MUN	14.5 for low NPN concentration	18.3 for high NPN concentration
		UUN:MUN	10.9 for low NPN concentration	14.1 for high NPN concentration
Broderick and Reynal (2009)	High proportion of RDP* originating from urea (0.35) vs. low percentage of RDP originating from urea (0.00)	UN:MUN	22.3 for high proportion of RDP originating from urea	28.2 for low proportion of RDP originating from urea
		UUN:MUN	16.5 for high percentage of RDP originating from urea	20.1 for low percentage of RDP originating from urea
Utley et al. (1970) †	0.6 × ad libitum water intake vs. 1.0 × ad libitum water intake	UN:PUN§	3.1 for 0.6 × ad libitum water intake	4.4 for 1.0 × ad libitum water intake
		UUN:PUN§	1.9 for 0.6 × ad libitum water intake	2.3 for 1.0 × ad libitum water intake
Weeth and Lesperance (1965) ‡	1.5 × ad libitum water intake vs. 1.0 × ad libitum water intake	UUN:PUN§	3.4 for 1.0 × ad libitum water intake	3.8 for 1.5 × ad libitum water intake

*Rumen degradable protein. †Experiment was carried out with Aberdeen Angus steers (c. 250 kg live weight).

‡Experiment was carried out with yearling Hereford heifers (live weight unknown).

§Because of the close relationship between PUN and MUN, observed differences in UN:PUN and UUN:PUN ratios are expected to yield similar differences in UN:MUN and UUN:MUN ratios as well.

Physiological Factors

Diurnal variation in PUN and MUN

The distribution of feed intake during a day affects diurnal variation in concentrations of rumen ammonia, PUN, MUN, and differences between PUN and MUN (Gustafsson and Palmquist, 1993). After a meal, microbial degradation of dietary protein is likely to cause an increase in the concentration of rumen ammonia which, due to transport of rumen ammonia to the blood and the subsequent conversion of blood ammonia into urea by the liver, is followed by a rise in PUN and, due to diffusion between milk and blood, in MUN. However, diets with a high rapidly degradable carbohydrate fraction may result in a decrease of rumen ammonia immediately after feeding, because degraded protein and rumen ammonia is utilized for microbial protein synthesis. The diurnal variation in MUN is of interest when MUN is used as an estimator of UUN, in particular because UUN is expressed on a daily basis whereas in practice, milk samples for MUN are obtained during milking only. Gustafsson and Palmquist (1993) observed that peak values of PUN may be up to 80 % higher than the lowest values and that MUN equilibrated with PUN with a lag time of 1 – 2 h, possibly caused by diffusion from blood to milk. Likewise, Carlsson and Bergstrom (1994) observed diurnal differences in MUN of up to 60 %. Thus the UUN:MUN ratio may differ depending on factors such as time of sampling milk and the feed intake pattern that is discussed in a following section on management factors.

Broderick and Clayton (1997) compared MUN and UUN during daytime (04:00 – 16:00 h) and night time (16:00 – 04:00 h). Average MUN during daytime (16.0 mg N/dL) was 33 % higher than during night time (12.0 mg N/dL) whereas UUN was even larger in the night time period. These differences between day and night resulted in UUN:MUN ratios of 4.6 and 7.7 for the daytime period and night time period, respectively. These differences in UUN:MUN ratios show that the relationship between MUN and UUN is not a constant. The fact that cows consume most of their diet during the daytime (Tolkamp et al., 2002; De Vries et al., 2003; Taweel et al., 2004) might explain the diurnal variation in MUN and UUN:MUN ratios.

In conclusion, the large diurnal variation in MUN and UUN:MUN ratios observed in some studies show that the UUN:MUN ratio is probably affected by the moment of sampling milk.

Labile N-reserves

According to Munro (1964) it was Carl Voit in 1866 – 67 who established the theory of labile N-reserves. He found that if N-intake was altered from a high to a low level, a net loss from the body N-pool occurred before a new equilibrium was established. This net loss of N was called the labile N-pool. This labile N-pool, as influenced by feeding management and between meal periods, may affect MUN and the expected UUN:MUN ratio as well. Approximately 0.05 of total body protein in Holstein dairy cows is considered to be highly labile, whereas total protein reserves may be as large as 0.50 of total body protein (Botts et al., 1979). Biddle et al. (1975) reported a labile body protein reserve in growing Jersey steers

of 0.056 of total body protein. The highly labile N is found mainly as urea in blood plasma and as protein in visceral organs such as the liver, kidney and intestinal epithelia, characterized by the highest protein turn-over rates in the whole body (Bannink et al., 2006). Some of these pools serve as protein donors to the body during pre-prandial periods.

This buffering effect of the liver, and possibly the intestinal cell wall, probably reduces peaks in PUN and MUN that would otherwise be expected after a meal due to postprandial deamination and oxidation of absorbed protein and diffusion of rumen ammonia to the blood. Peaks of PUN after a meal are mainly caused by diffusion of ammonia from the rumen to the blood whereas part of the postprandial protein absorbed by the small intestine is probably stored as protein in liver and gut tissues.

There is some data available with respect to short time (24 – 48 h) oscillation in dietary protein intake and PUN. Ludden et al. (2002b) observed that wether sheep receiving an oscillating protein diet that was switched over every 48 h from 130 to 170 g crude protein (CP)/kg dry matter (DM) and vice versa had lower serum urea-N concentrations (blood samples taken every 4 h during 4 days) than wethers receiving a constant daily 150 g CP/kg DM ration (serum urea of 12.1 vs. 13.4 mg N/dL, respectively), despite a similar average N-intake and dietary CP digestibility for the two treatments. In other experiments with lambs, however, Cole (1999) found no clear differences in PUN between a control diet (125 g CP/kg DM) and oscillating diets that were switched every 24 or 48 hours between high (150 g CP/kg DM) and low (100 g CP/kg DM) protein diets (based on blood samples taken before the morning feeding). Archibeque et al. (2007) also found no significant differences in PUN between an oscillating diet (dietary CP content oscillated every 48 h between 99 and 142 g CP/kg DM) and a daily 125 g CP/kg DM diet in an experiment with growing wethers, although the dietary N-intake was 14.4 % higher (25.4 g N/d) for wethers receiving the oscillating protein diet than for the wethers receiving the daily 125 g CP/kg DM diet (22.2 g N/d).

The results described above indicate that there is no clear relationship between PUN (and subsequently MUN) and oscillation of dietary protein intake, which might be caused by the buffering capacity of the body in fixating absorbed protein and gradually releasing this fixated protein later on. This theory is further strengthened by results of Ludden et al. (2002a), who found that wether lambs receiving a diet that oscillated in CP from 130 to 170 g CP/kg DM every 48 h had organ masses that were 5.4, 17.3, and 6.2 % higher for the liver, small intestine, and reticulorumen, respectively, than lambs receiving a daily 150 g CP/kg DM diet, despite a similar average daily N-intake. Kiran and Mutsvangwa (2009) observed an increased N-retention in growing lambs fed oscillating protein diets over 24 days, which was probably due to an increase in organ mass of the gastro intestinal tract and liver. Adaptation (by enlarging labile protein tissue) to periods of protein deficiency may explain the observed increase in organ mass of liver and gastro intestinal tract in lambs receiving oscillating protein diets.

In conclusion, although it is not possible to quantify the effect of the buffering capacity of the labile protein pool on diurnal changes in PUN, MUN, and UUN:MUN ratio, it is likely that the fluctuations in PUN and MUN before and after a meal are different from the values expected from the postprandial absorption of protein and ammonia from the GIT.

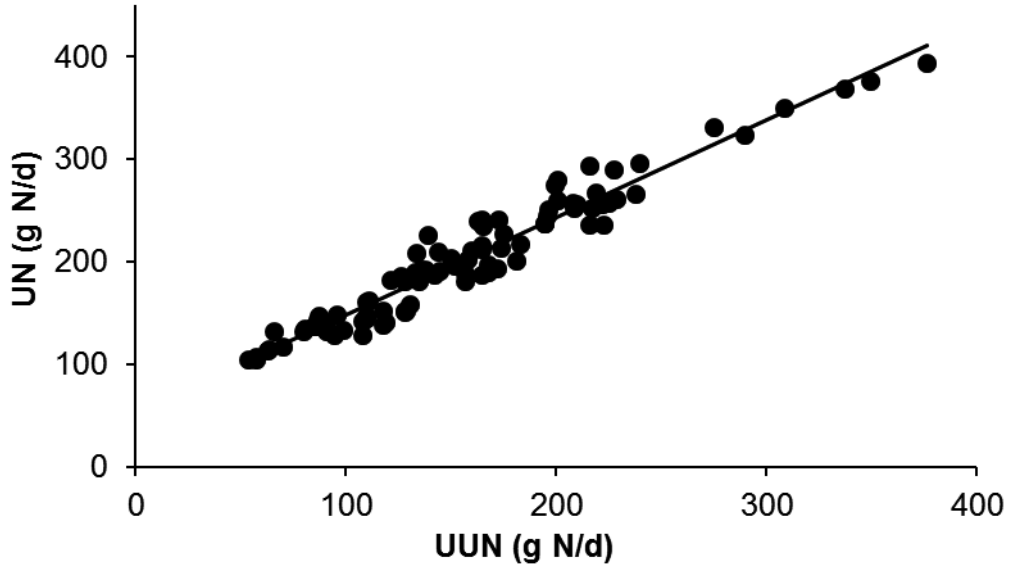

Figure 2. Simple regression of urinary nitrogen excretion (UN; g N/d) on urinary urea nitrogen excretion (UUN; g N/d). UN = 51.9±4.42 + 0.95±0.026 × UUN, R^2 = 0.94. The model was derived from 84 observations collected in 20 experiments (references available on request).

Renal urea reabsorption

A number of studies consider a linear relationship between PUN (or MUN) and UN (Jonker et al., 1998; Kauffman and St-Pierre, 2001; Kohn et al., 2002). However, there are indications that renal urea reabsorption may be actively regulated by means of urea transporters (UT), depending on certain conditions such as protein intake or dehydration status. This active regulation of renal urea absorption is likely to affect UUN:MUN ratios. Two groups of urea transporters have been identified in mammals, UT-A and UT-B (Bagnasco et al., 2000), which offer the possibility of a rapid transfer of urea across cell membranes. In lambs, UT-B is present in tissues of the rumen, liver, kidney, small intestine and caecum whereas UT-A is present in tissues of the kidney, liver and duodenum (Marini et al., 2004). Inoue et al. (2005) showed that the presence of UT-B in the inner and outer medulla of kidneys from rats decreased significantly when offered a diet with high instead of low protein content. A larger quantity of UT-B in the outer and inner renal medulla of rats fed a low protein diet can explain the often reported increase of urea recycling in rats (Smith et al., 1995; Sands et al., 1996; Kato and Sands, 1999). Tsukaguchi et al. (1998) showed the existence of three UTs in the rat kidney and

concluded that one of these UTs was responsible for the bulk of urea reabsorption in the terminal inner medullary collecting duct.

The possibility of active urea transport has been shown by Isozaki et al. (1993) and Sands et al. (1996). Sands and colleagues found that active urea transport took place in the inner medullary collecting duct of rats fed a low protein diet for 3 weeks. This transport of urea was blocked in the absence of sodium, showing that a secondary active sodium transporter was coupled with transport of urea. Schmidt-Nielsen et al. (1957) found that the PUN level in a young camel during a period of water deprivation was significantly higher than that in an older camel and explained this difference in PUN by assuming that the young, still growing camel saved and stored the urea for growing purposes. This would mean that urea excretion by the kidney is not only controlled by PUN alone, as assumed in a number of studies, but also by the physiological status or hormonal state of the animal. If the (active) renal reabsorption of urea in low protein diets observed in rats occurs as well, then this will probably result in a distortion of the general linear relationship between PUN and UUN and as a result the UUN:MUN ratio (as MUN is a reflection of PUN) is likely to change at low PUN values. More information on the effect of dietary protein on UUN:MUN ratio is presented in a following section on nutritional factors. Besides an increase in renal reabsorption of urea in order to minimize excretion of N, Eriksson and Valtonen (1982) observed in experiments with goats fed high (140 g/kg DM) or low (< 20 g/kg DM) CP diets that UN on the low CP diet was minimized by means of a combination of a lowered PUN, an increased fractional renal reabsorption rate of urea, and a decreased glomerular filtration rate as compared with the high CP diet. The UUN:PUN ratio in the study of Eriksson and Valtonen (1982) was 0.57 and 0.04 for the high and low CP diet, respectively.

Renal urea recycling is also influenced by the hydration status of the animal. Bagnasco et al. (2000) found that expression of UT-A mRNA was especially increased in the inner medulla of water deprived rats compared to control rats. Urea is an important osmolite in the renal reabsorption of water. An increase in UT-A transporters during dehydration may indicate an increase in urea reabsorption in order to increase renal osmotic pressure, resulting in increased water absorption from the filtrate, but also in an increase in PUN (and subsequently MUN due to diffusion of urea between blood and milk). It has been shown in a number of studies that dehydration results in increased levels of PUN and/or MUN (Weeth et al., 1967; Utley et al., 1970; Steiger Burgos et al., 2001). More quantitative information on the effect of water intake on UUN:MUN ratio is given in a following section on nutritional factors.

In conclusion, renal urea filtration is a regulated process that depends on, amongst others, the hydration status of the animal and the protein supply to the animal. Hence, level of protein and water supply, and their interaction, may affect the relationships between MUN and UUN.

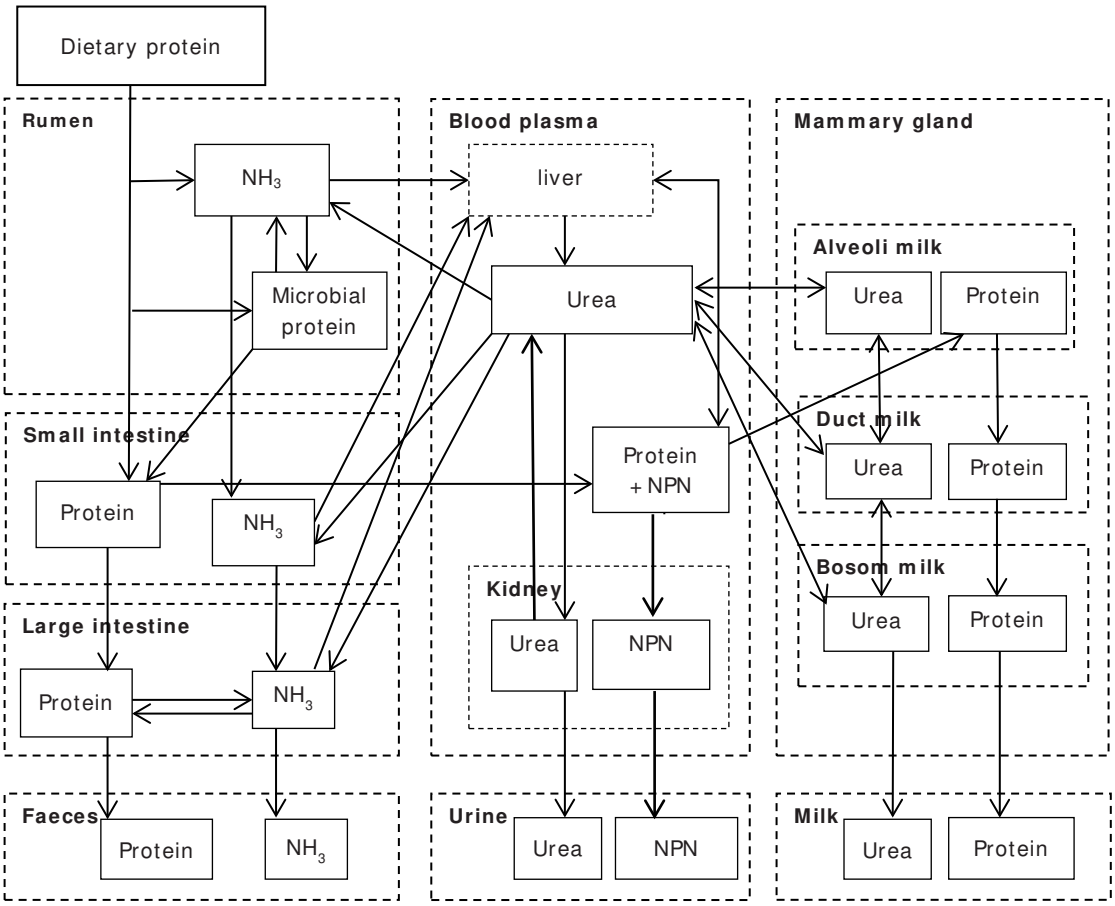

Figure 3. Overview of nitrogen flows in the lactating ruminant that influence levels of urea in blood plasma and milk, and possibly affect the relationship between urea concentration in the milk and the quantity of nitrogen excreted in the urine. Arrows represent fluxes and boxes enclosed by solid lines represent pools. NPN is defined as non-protein-N with the exception of urea.

Biological rhythm

Daily rhythmicity is a well-known feature of mammalian species and daily rhythmicity may affect levels of PUN, MUN, and UUN:MUN ratio. The suprachiasmatic nucleus in the hypothalamus is the dominant regulator of circadian rhythmicity, influenced by light. It has been shown in rats during restricted feeding conditions that, besides the suprachiasmatic nucleus, other food-entrainable circadian oscillators are present in the digestive organs (Davidson et al., 2003). Takata et al. (2002) showed that mRNA levels of mPer2, a clock gene, in mice liver had a significant diurnal variation suggesting that liver activity is time-regulated, although not necessarily independent of feeding time.

It was suggested by Piccione et al. (2003) that the rhythm of PUN concentration in goats was driven by digestive processes and not by time of feeding. This contrasts, however, with the studies of Lefcourt et al. (1999) and Piccione et al. (2007) in dairy cattle in which PUN

increased prior to feeding, which was probably not caused by feed intake and day and night rhythm alone. Piccione et al. (2007) conducted an experiment with Italian Brown cows with access to feed at either 08:00 or 16:00 h for 2 h. In both treatments, PUN concentrations increased prior to feeding to around 2.8 mg N/dL. This increase was independent of day and night periods because it occurred in both groups. The increase in PUN prior to feeding observed by both Piccione et al. (2007) and Lefcourt et al. (1999) may be explained by an increase in urea formation by the liver from the labile protein reserve, a reduction of urea excretion by the kidneys, a reduction in blood plasma volume due to the excretion of liquids into the digestive tract, an increase in water excretion into the urine, or other processes.

With respect to hydration status, Buemi et al. (2007) found that body water content of humans showed a circadian rhythm being highest between 09:00 and 23:00 h. There is a possibility that a comparable circadian rhythm of glomerular filtration rate and body fluid content exists in dairy cows which affects PUN, and that the circadian rhythms of PUN observed in the experiment of Piccione et al. (2007) increased PUN prior to feeding.

The existence of feed intake independent diurnal variation in PUN was shown by Piccione et al. (2003). In an experiment with feed-deprived goats, diurnal variation appeared to remain during 2 consecutive days after the last feeding. During another experiment from Piccione et al. (2003) with the same goats, animals were deprived of feed during 3 days under a continuous lighting scheme in which diurnal variation of PUN was less compared to the situation of feed deprivation with alternating dark and light phases. These results indicate that circadian rhythms, influenced by both day and night rhythm and feeding time, influence PUN.

In conclusion, circadian patterns most likely influence diurnal PUN, and as a result MUN. The circadian pattern of MUN may result in different UUN:MUN ratios depending on the time of milking. However, information with regard to the effect of clock genes on diurnal variation in PUN and MUN, as influenced by time and frequency of milking and feeding and dark and light periods, seems lacking for dairy cows.

Management Factors

Feeding frequency and milking interval

Shabi et al. (1998) found that a feeding frequency of twice vs. four times a day resulted in 4.4 mg N/dL lower PUN at the time of milking. Cows were fed twice a day at the time of milking for the ‘twice feeding per day treatment’ whereas for the ‘four feedings per day treatment’ cows were fed during milking and in between two milkings. However, levels of PUN at 2 and 4 h after feeding were lower for the cows fed four times a day. Because of the strong positive correlation between PUN and MUN (Broderick and Clayton, 1997) and the rapid (within 1 h) equilibration between PUN and MUN (Gustafsson and Palmquist, 1993), it appears that MUN and subsequently UUN:MUN ratio may be manipulated substantially by changing the feeding frequency, assuming an unaltered feed intake and N-metabolism.

It was shown by Nielsen et al. (2005) that cows milked at 6-h intervals had higher MUN values than cows milked at 12-h intervals (11.3 and 9.9 mg N/dL, respectively). Average fat concentration from the cows milked at the 6-h interval was also higher compared with the cows milked at the 12-h interval while protein content did not change substantially. The increase in milk fat content for the 6-h treatment, however, could decrease MUN only slightly, circa 0.09 mg N/dL, due to a reduced volume of urea space per volume unit of milk. Increased MUN values at shorter milk intervals were also observed by Friggens and Rasmussen (2001), who found a decrease in MUN of 0.18 mg N/dL for every extra hour the milking interval was increased compared to the average milking interval. Hale et al. (2003), however, found no differences in MUN at 6 or 12-h milking intervals during the first 3 weeks of lactation.

Explanations for higher MUN at shorter milking intervals are speculative. Nielsen et al. (2005) suggested that the time of feed intake, relative to the moment of milking and milk sampling, could explain this trend of higher MUN values at shorter milking intervals. For example, it was shown in research from De Vries et al. (2003) that there were clear peaks of feed alley attendance just after milking. Based on the results of Gustafsson and Palmquist (1993) and Ikuta et al. (2005), MUN peaks circa 4 h after a meal. Therefore, the explanation of Nielsen in the case of a 6-h milking interval seems reasonable. However, feed intake pattern was not recorded in the aforementioned studies on milking intervals. Another explanation might be that an increase in milking frequency is probably accompanied with an increase in milk production and dry matter intake (DMI), resulting in an increased dietary N-intake and an increase in the level of PUN and subsequently MUN.

In conclusion, assuming an unaltered dietary intake of N and excretion of UUN, the UUN:MUN ratio is affected by the frequency of milking and feeding.

Within-day synchronization of protein and energy

Although feeding total mixed rations (TMR) to dairy cows is quite common, a large number of farmers still offer roughage and concentrates separately to cows (as is the case when cows are allowed to graze). This influences the instantaneous amount of feed protein degraded in the rumen and subsequently the height and duration of rumen ammonia concentrations and of PUN. As a result, the separation of dietary energy and protein consumption in time might affect MUN and the UUN:MUN ratio, depending on the time of milking.

Ikuta et al. (2005) supplemented half of the concentrates 30 min after feeding the basal diet, whereas the other half of the concentrates was supplemented 1.5 h after feeding the basal diet. Concentrates were separated into grain-rich and protein-rich parts. Rations were isonitrogenous and isocaloric and DMI and milk production between treatments was similar. Lower concentrations of rumen ammonia, PUN, and MUN were measured during the day in the cows receiving the grain supplement before the protein-rich supplement compared to cows receiving the protein-rich supplement first, or cows receiving the protein-rich and energy-rich supplement combined. The values of PUN and MUN of the group receiving the grain supplement first were

circa 2.0 mg N/dL lower than for the group receiving the protein supplement first. It is unclear, however, whether this decrease in MUN also caused a different UUN:MUN ratio because UUN was not measured.

For an identical grass/maize silage diet, Geerts et al. (2004) tested whether feeding this diet as a mixed grass/maize silage diet or feeding the maize silage in the morning and the grass silage in the evening (after the evening milking) would affect MUN. As expected, MUN in the evening milk was 2.0 mg N/dL higher for the TMR group compared to separate feeding whereas it was 2.0 mg N/dL lower in the morning milk. Combining the morning and evening milk resulted in a similar MUN for both groups.

Kim et al. (1999) tested the effect of intraruminally infusing 2.0 kg of maltodextrin in three time intervals: 1) continuous infusion, 2) two infusion intervals of 6 h each starting at 10:00 and 22:00 h, and 3) two infusion intervals of 6 h each starting at 16:00 and 04:00 h. The basal diet (196 g CP/kg DM) was supplied in two equal portions at 10:00 and 22:00 h. In comparing the three infusion time intervals, it appeared that time interval 2, consisting of two infusion intervals of 6 h each starting at 10:00 and 22:00 h, resulted in a 1.0 mg N/dL higher MUN (MUN = 17.4 mg N/dL) compared to the other 2 infusion intervals and a slightly lower UN:MUN ratio (UN:MUN = 7.6) compared to the UN:MUN ratios for time interval 1 and 3 (UN:MUN ratios of 7.9 and 8.3, respectively).

In conclusion, the results indicate a small effect of within-day dynamics of N-metabolism on diurnal changes in PUN, MUN, and on UN:MUN ratio. However, solid evidence is lacking and more information is required on the effect of (within day) synchronization of rumen fermented protein and energy on and levels of PUN and MUN and ratio of UUN:MUN.

Cow Factors

Body weight

Body weight (BW) is positively correlated with excretion of UN and UN:MUN ratio (Kauffman and St-Pierre 2001). This makes sense because at a similar PUN a large animal has a larger pool of blood urea than a small animal, due to the larger urea space volume. Therefore, a reduction in PUN in a large animal requires the excretion of more urea into urine compared to a smaller animal. Kauffman and St-Pierre (2001) found linear relationships between MUN and UN that were different for Jersey and Holstein cows. Per mg N/dL of MUN, 17.6 g N/d of UN was excreted for Holstein cows compared to 11.8 g N/d for Jersey cows. When BW was included in the model, the effect of breed on UN:MUN ratio was not significant. Furthermore, a within-breed effect of BW on UN:MUN ratio was shown by Kohn et al. (2002). The addition of BW next to MUN in the model resulted in a reduced root mean square prediction error of 33 %. Likewise, Zhai et al. (2007) found in an experiment with lactating Chinese Holstein cows an increased model fit (R^2 increased from 0.82 to 0.99) when, next to MUN, BW was included in the UN prediction model.

Reported regression coefficients of BW × MUN interaction on UN vary slightly: 0.0259 (Kauffman and St-Pierre, 2001; Kohn et al., 2002), 0.0283 (Wattiaux and Karg, 2004), and 0.0247 (Zhai et al., 2007). It has been suggested that these differences in regression coefficients were caused by differences between studies in the analytical method of MUN determination and/or UN ranges on which the regression coefficient is based (Wattiaux and Karg, 2004), or by differences in breeds (Zhai et al., 2007).

In conclusion, the factor BW affects the relationship between UN and MUN.

Genetics

A number of studies report heritability estimates for MUN varying from 0.13 – 0.14 (Stoop et al., 2007; Konig et al., 2008; Bastin et al., 2009), 0.15 – 0.22 (Mitchell et al., 2005) and 0.44 – 0.59 (Wood et al., 2003). However, information about the correlation between the breeding value for MUN and the efficiency of N-utilization or UN is scarce. Šebek et al. (2007) examined the relationship between breeding value for MUN and efficiency of N-utilization, using a dataset of 15,720 animal week records, originating from 723 cows from 26 experiments. The model used to calculate the breeding values was corrected for factors including parity, days in milk, location, diet, and season. Breeding values for MUN ranged from – 2.3 to 2.8 mg N/dL but no significant relationship could be established between breeding value of MUN and observed efficiency of N-utilization. The range in breeding values for MUN and the absence of a relationship between breeding value of MUN and observed N-efficiency may be explained by physiological differences between cows with respect to the dynamics of N-intake, N-digestion, efficiency of N-utilization, renal functioning or urea diffusion rates. Excretion of UUN was not measured in the study of Šebek et al. (2007), which makes it impossible to draw a conclusion about the effect of genetics on the relationship between UUN and MUN in this study. Vallimont et al. (2011) estimated a heritability of 0.57 for MUN. The genetic correlation with efficiency of N-utilization was 0.10 and was not significantly different from zero. On the assumption that a reduced efficiency of N-utilization is largely associated with increased excretion of N in urine (Kebreab et al. (2002), this absence of genetic relationship indicates that high breeding values for MUN do not affect efficiency of N-utilization. Together, the results of Šebek et al. (2007) and Vallimont et al. (2011) suggest that genetic differences in breeding values for MUN are not related to efficiency of N-utilization and do affect the ratio between UUN and MUN, but more research on this relationship is required.

In conclusion, MUN is moderately heritable but breeding value of MUN is unrelated to efficiency of N-utilization and probably to UUN as well.

Nutritional Factors

Water intake

The intake level of water affects PUN and subsequently MUN (Weeth and Haverland, 1961; Weeth and Lesperance, 1965; Steiger Burgos et al., 2001), even when N-excretion in urine and

feces did not change (Utley et al., 1970). With respect to a restriction in water intake, Schmidt-Nielsen et al. (1957) observed a four-fold increase of PUN in camels during a water restricted period, whereas the amount of urea excreted in urine remained unchanged. From these results it was concluded that urea excretion via the urine is a regulated process, which depends on the need of the animal to preserve urea. In a study from Steiger Burgos et al. (2001), dairy cows receiving 0.50 of voluntary water intake had a 1.58 higher MUN than the control group receiving water ad libitum. Unfortunately, no information was given with respect to N-intake and N-excretion in the study of Burgos et al. (2001), although it is reasonable to assume that a restriction in water intake will not increase N-intake (Little et al., 1976; Silanikove, 1985; Maloiy et al., 2008) and that the increase in MUN could not be explained by an increase in dietary N-intake.

An increase in water intake due to an increase in salt intake has the opposite effect on PUN as compared to water restriction. In growing Hereford heifers, Weeth and Haverland (1961) observed a decrease in PUN of 27 % when water intake was increased (by means of saline water) by 48 % from 29 to 43 liters. Likewise, Weeth and Lesperance (1965) found in a study with Hereford heifers that an increase in water consumption of 52 % reduced PUN concentration by 14 % at a low dietary protein ration (120 g CP/kg DM) and at a high dietary CP ration (210 g CP/kg DM) PUN was reduced with 4 % when water intake was increased with 34 %. In a study with sheep, Godwin and Williams (1984) observed a reduction in PUN from 68.0 to, on average, 36.3 mg N/dL when water intake was increased by 32 % from 1.7 to 2.3 L/d However, Ergene & Pickering (1978) found no differences in PUN in sheep when urine production was increased upon supply of saline (10 g NaCl/L) drinking water.

Based on the studies mentioned in this chapter it seems that increased water intake lowers PUN whereas restricted access to water increases PUN and subsequently MUN. This effect of water intake on MUN is likely to affect the UUN:MUN or UN:MUN ratios as well in dairy cattle receiving feed rations that vary in mineral contents.

Rations rich in minerals such as sodium (Na) and potassium (K) lead to increased water consumption and urine production (Bannink et al., 1999), thereby decreasing PUN and consequently MUN. Mineral contents of grass silage harvested from N and K fertilized grasslands are high compared to the mineral contents in maize silage. For example, average concentrations of K and Na in grass silages in the Netherlands are 34.1 and 2.3 g/kg DM, respectively whereas average concentrations of K and Na in maize silage are substantially lower, viz. 12.0 and 0.2 g/kg DM (CVB, 2007). As a consequence, grass-based rations probably result in a high consumption of K and Na compared to maize-based rations, leading to higher urine production, resulting in lower levels of PUN and MUN and consequently higher UUN:MUN ratios. De Campeneere et al. (2006) and Van Duinkerken et al. (2005) studied the effect of roughage type (maize silage vs. grass silage) on MUN and rates of ammonia emission from barns. In comparison to the maize silage diet, De Campeneere et al. (2006) observed a 143 % higher urine production for the grass silage diet, whereas Van Duinkerken et al. (2005)

estimated 60 % higher urine production based on a regression equation of Bannink et al. (1999) with the grass silage diet. A lower MUN was found for the grass silage rations, which might be attributed in part to the increase in urine production. In the study of De Campeneere et al. (2006), UN:MUN ratios were 11.3 and 14.1 for the maize and grass based rations, respectively. However, confounding factors like differences in DMI and dietary protein intake, and extent of rumen hind gut fermentation, prohibit the drawing of solid conclusions.

Schmidt-Nielsen et al. (1958) studied the effect of rising or decreasing urine production rates (at equal dietary N-intake) by means of an intravenous mannitol or glucose infusion on UUN and observed that during periods of rising urine flow UUN was increased by 35 %, whereas during periods of decreasing urine flow UUN was decreased until a steady state urine flow was reached again. The temporary increase in UUN during a rising urine flow results in a decreased urea blood plasma pool and, as a consequence, a reduced level of MUN. This mechanism of temporary change in UUN during periods of increasing or decreasing urine flows might actually be responsible for the difference in MUN levels found in the previously described experiments from Steiger Burgos et al. (2001), Van Duinkerken et al. (2005), and De Campeneere et al. (2006).

The negative correlation between MUN and urine production may be explained by the fact that renal blood flow is increased at increasing water intake and urine production, and secondly by the fact that urea is used as an osmolite in the kidneys to concentrate urine. At high urine production there is less need to concentrate urine, and urea concentrations in the interstitial cells of the inner medulla may fall (Rabinowitz and Gunther, 1974). A low urea concentration in the inner medulla probably results in low quantities of urea transported back to the blood circulation by the ascending vasa recta located in the inner medulla in the kidney, whereas the opposite might happen during periods of low urine production.

In conclusion, both water restriction and increased water intake affects PUN, MUN, and UN:MUN ratio, with water restriction having the highest impact on levels of PUN, MUN and UN:MUN ratio.

Dietary protein concentration

In a previous section it was shown that the dietary concentration of protein affects the expression of UT proteins in the inner medullary collecting duct of the kidneys. This altered expression is likely to affect renal urea reabsorption, the relationship between PUN and UUN, and as a result the relationship between MUN and UUN. For example, Olmos Colmenero and Broderick (2006) tested the effect of dietary CP on milk production by varying the dietary CP content from 135 to 194 g/kg DM: the UUN:MUN ratio increased from 8.2 at 135 g CP/kg DM to 13.3 at 194 g CP/kg DM. The results of Olmos Colmenero and Broderick (2006) were confirmed in a study from Borucki Castro et al. (2008) in which UUN:MUN ratio increased from 10.2 at 162 g CP/kg DM to 13.5 at 201 g CP/kg DM.

Figure 4 shows the results from a meta-analysis, based on 23 trials, in which the relationship between dietary CP concentration and the UUN:MUN ratio was studied. The linear plateau relationship that was found indicated an increased renal reabsorption rate of urea (possibly combined with a decreased glomerular filtration rate) at decreasing dietary CP concentrations (lower than 170 g CP/kg diet DM), whereas at dietary protein levels exceeding 170 g CP/kg DM the UUN:MUN ratio did not change, indicating an unchanged renal reabsorption rate of urea.

In conclusion, the level of dietary CP affects the UUN:MUN ratio and the relationship between dietary CP concentration. The UUN:MUN ratio can be described by a quadratic broken stick model.

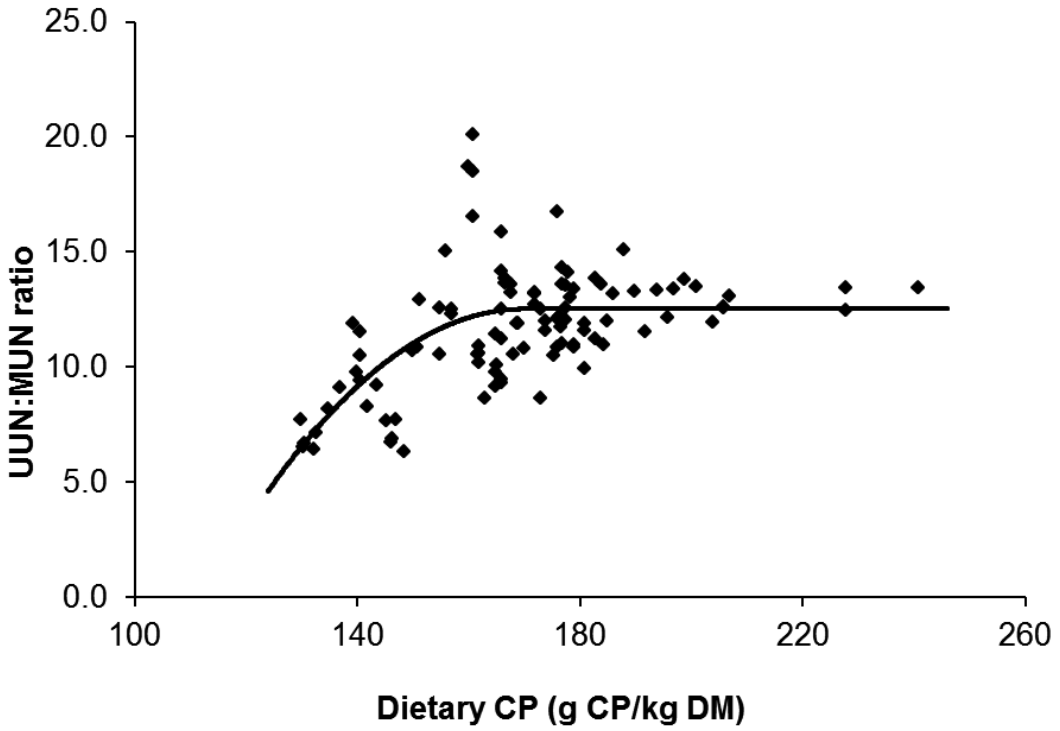

Figure 4. Relationship between dietary content of crude protein (CP; g CP/kg dry matter (DM)) and the ratio between urinary urea nitrogen excretion (UUN; g N/d) and milk urea nitrogen concentration (MUN; mg N/dL). A quadratic broken line model yielded the best model fit. The model: UUN:MUN = $L + U \times (R - CP)^2$, where $(R - CP)^2$ is zero at values of CP above a threshold crude protein content R (g CP/kg DM). The model is adapted from Robbins et al. (2006). The value of L as the maximum UUN:MUN was 12.5±0.28, and the value of U as the decline parameter was –0.374±0.1822, and the threshold value of R as CP at which the maximum UUN:MUN is reached was 17.0±0.82. The model was derived from 102 observations collected in 23 experiments (references available on request).

Non- urea-N-excretion in urine

High UN:MUN ratios might be expected at low levels of PUN, MUN, and intake of CP because the quantity of nitrogen excreted in urine, excluding urea nitrogen (NUUN; g N/d) excreted in urine is, probably largely unrelated to urea levels in the blood and milk. The quantity of NUUN can be calculated as the intercept value from MUN - UN regression formula. Intercept values between 25 and 56 g N/d have been reported (Ciszuk and Gebregziabher, 1994; Gonda and Lindberg, 1994; Zhai et al., 2005; Zhai et al., 2007). Another way of estimating NUUN is to take the intercept value of the regression equation where UN is regressed on UUN (Fig. 2), yielding in the current study a value of 51.9 g N/d.

An exponential relationship between NUUN and MUN was observed based on a meta-analysis of results from 20 trials (Fig. 5) in which the NUUN:MUN ratio is negatively related to MUN. This negative relationship between the NUUN:MUN ratio and MUN should be considered when UUN is estimated based on data from UN and MUN. Bristow et al. (1992) found that the main N-constituents in cattle urine were urea (0.70), allantoin (0.08), hippuric acid (0.06), creatinine (0.04), creatine (0.03), and ammonia (0.03). An increase in DMI (and fermentable organic matter) is likely to result in increased rumen microbial protein synthesis and an increase in NUUN due to an increase in excretion of purine derivatives (mainly allantoin derived from rumen microbial nucleic acids) and hippuric acids (derived from plant phenolic cinnamic acids). Hence, an increase in DMI is likely to increase NUUN excretion, although its effect on total UN secretion will remain small considering the small contribution of hippuric acids and allantoin to UN.

In conclusion, a decrease in MUN will be associated with an increased NUUN:MUN ratio. This fact should be considered when estimating the excretion of UUN based on UN.

Rumen pH and concentration of carbon dioxide, butyrate, and ammonia

Fast vs. slow rumen fermentable carbohydrate diets have been shown to affect the flux of urea from blood to rumen liquid across the rumen wall (Kennedy et al., 1981; Norton et al., 1982). This effect of type of carbohydrate on urea flux might affect the ratio of UUN:MUN as well. Rumen fermentation of ingested feed yields fermentation products including short chain fatty acids, ammonia, and carbon dioxide (CO_2). High production rates of short chain fatty acids after a meal result in a lowered rumen pH. Studies show that rumen pH, and concentrations of CO_2 and butyrate are correlated with the transport rate of ammonia and urea across the rumen wall (Thorlacius et al., 1971; Rémond et al., 1993; Abdoun et al., 2005; Abdoun et al., 2010). For example, Abdoun et al. (2005) observed that ammonia fluxes across cell walls of isolated rumen epithelia of sheep at equal ammonia levels decreased when mucosal pH was lowered from 7.4 to 6.9 and from 6.9 to 6.4. In another study from Abdoun et al. (2010) on urea transport across isolated rumen epithelia of sheep, the effect of rumen pH on the net transfer of blood urea across the rumen wall of sheep was found to be highest at pH 6.2 and would decrease rapidly at higher or lower pH values. Besides the effect of rumen pH, it has been shown in a number of

studies that ruminal concentrations of CO_2 and/or butyrate stimulate urea transport from blood to rumen (Thorlacius et al., 1971; Norton et al., 1982; Rémond et al., 1993; Abdoun et al., 2010). It has been suggested that the effect of CO_2 on the flow of blood urea across the rumen is caused by an increase in the blood flow to the rumen epithelium; however, no relationship was found in the study of Dobson et al. (1971) between rumen blood flow and urea transport. Furthermore, the pure unconfounded effect of blood flow on ammonia transport from the rumen to the blood has not yet been elucidated.

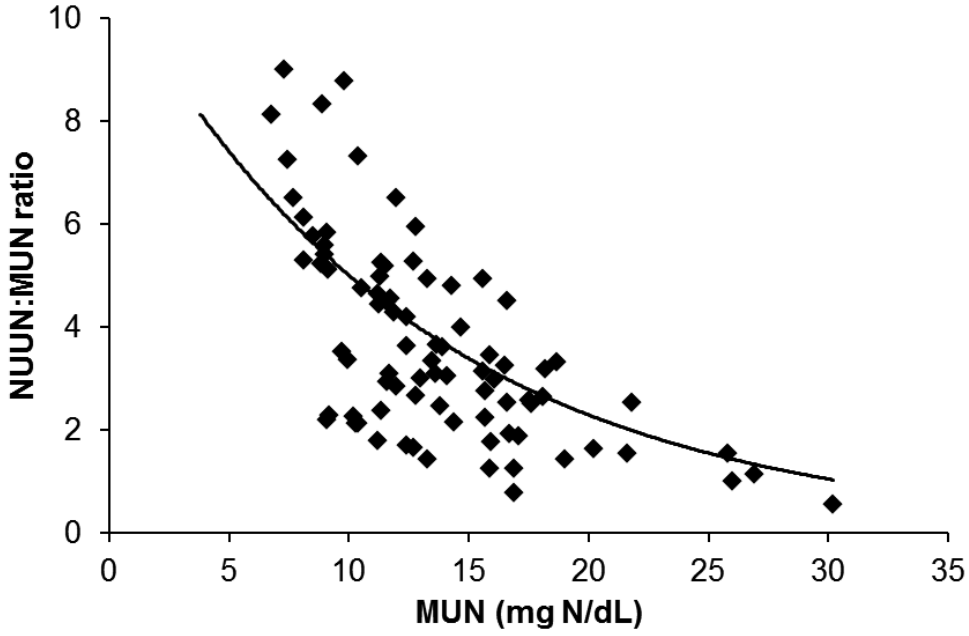

Figure 5. Relationship between milk urea nitrogen concentration (MUN; mg N/dL) and the ratio between nitrogen excretion in urine excluding urea nitrogen (NUUN; g N/d) and MUN. An exponential model yielded the best model fit. NUUN:MUN ratio = 13.68±2.067 × exp(–0.104±0.0133 × MUN). The model was derived from 84 observations collected in 20 experiments (references available on request).

Another factor that affects blood-rumen urea transport is the concentration of rumen ammonia. Cheng and Wallace (1979) found a negative correlation between rumen urease activity and rumen ammonia concentration and suggested a mechanism of decreased urea transport at increased concentrations of rumen ammonia. Such a negative correlation between urease activity in the rumen (epithelium) and urea transport across the rumen wall could explain the up to 13-fold increase of urea transport across the non-rinsed rumen wall versus the water-rinsed rumen wall in the study of Houpt and Houpt (1968), or the negative correlation between rumen ammonia level and urea transport across the rumen wall found in the studies of Kennedy et al. (1981) and Rémond et al. (1993).

Hristov et al. (2005) tested the effect of type of carbohydrate on rumen ammonia utilization and observed that starch- and glucose-rich diets resulted in lower concentrations of rumen ammonia,

PUN, and MUN compared to a neutral detergent fibre (NDF)-rich diet. The NDF-rich diet resulted in a lower UN:MUN ratio (UN:MUN = 15.1) than the starch- and glucose-rich diets (UN:MUN of 16.4 and 16.2, respectively). Similar results were found by Beckman and Weiss (2005) and Broderick (2003). This effect of type of carbohydrate on UN:MUN ratio could have been affected by rumen microbial protein synthesis and its effect on level of rumen ammonia.

In conclusion, experimental evidence indicates that urea transport across the rumen wall is subject to regulation, being stimulated after a meal when rumen concentrations of CO_2 and butyrate are high. Type of dietary carbohydrate will affect rate of fermentation and profile of short chain fatty acids and magnitude of ammonia levels in rumen fluid, and thus is likely to influence dynamics of PUN and thereby UUN:MUN ratio. However, the sole effect of rumen pH and ruminal concentration of CO_2 and butyrate on UUN:MUN ratio remains to be tested.

Analysis Methods

Variation in MUN within and between experiments might partly be attributed to the method of urea analysis. Differences between urea analysis methods and devices or labs may lead to systematic differences in MUN between studies. Peterson et al. (2004) compared a number of analytical devices and methods on the analysis of milk samples and the recovery of MUN from urea spiked milk samples. Recovery of MUN varied from 0.85 to 0.95 and the standard error (SE) varied from 0.028 to 0.076 for the enzymatic analysis methods (based on conversion of urea to ammonia by the enzyme urease) whereas for the infra-red-spectrometry methods recovery of MUN varied from 0.47 to 0.95 and the SE varied from 0.099 to 0.101. Also some disturbing effects of level of urea, fat, lactose and protein in the milk on recovery of MUN were found for both methods. Similar disturbing effects of level of urea, fat, and protein in the milk on analysis of MUN were observed by Kohn et al. (2004). Broderick (2003) regressed values of MUN determined with an enzymatic method on values of MUN determined with infra-red-spectrometry and found a low correlation coefficient of 0.20. Regression of data in the current study (not shown) between MUN values determined with an enzymatic method and values determined with infra-red-spectrometry yielded a correlation coefficient of 0.81 and it appeared that enzymatically determined MUN values smaller than 3.0 mg N/dL yielded MUN values of zero when analyzed by infra-red-spectrometry, indicating a high lower limit of detection for MUN by infra-red-spectrometry. Finally, in studies in the USA, systematic differences in MUN and UN:MUN or UUN:MUN ratios might be present between studies having their milk samples analyzed before and after 1998 due to a hardware defect in MUN analyzers of DHIA laboratories (Kohn et al., 2002).

In conclusion, the high variability of recovered urea measured by means of infra-red-spectrometry and the low correlation coefficients found between MUN analyzed enzymatically and MUN analyzed by infra-red-spectrometry indicates the necessity of analyzing MUN by means of an enzymatic method in order to obtain accurate data to be used to study the relationship between UUN and MUN.

Conclusions and Future Perspectives

A number of factors have been shown to affect the relationships between PUN, MUN, UUN, and UN. Factors such as body weight, water intake, urine production, dietary protein level, and time and frequency of feeding and milking are shown to affect the relationship between MUN and UUN. Accounting for the effects of these factors on the relationship between MUN and UUN can increase the applicability and accuracy of MUN as predictor of protein utilization efficiency and UUN, and may explain differences in MUN-UN regression formulas and their prediction results between studies. Due to the many factors that affect MUN, UUN, and the UUN:MUN ratio, a mechanistic modeling approach seems appropriate to predict the relationship between MUN and UUN.

Chapter 3

Prediction of urinary nitrogen and urinary urea nitrogen excretion by lactating dairy cattle in Northwestern Europe and North America: A meta-analysis

J.W. Spek[1,2], J. Dijkstra[1], G. van Duinkerken[2], W.H. Hendriks[1], and A. Bannink[2]

[1]Animal Nutrition Group, Wageningen University, PO Box 338, 6700 AH, Wageningen, The Netherlands
[2]Department of Animal Nutrition, Wageningen UR Livestock Research, PO Box 65, 8200 AB, Lelystad, The Netherlands

Journal of Dairy Science (2013) 96, 4310-4322

Abstract

A meta-analysis was conducted on the effect of dietary and animal factors on excretion of total urinary nitrogen (UN) and urinary urea nitrogen (UUN) in lactating dairy cattle in North America (NA) and Northwestern Europe (EU). Mean treatment data were used from 47 trials carried out in NA and EU. Mixed model analysis was used with experiment included as random effect and all other factors, consisting of dietary and animal characteristics, included as fixed effects. Fixed factors were nested within continent (EU or NA). A distinction was made between urinary excretions based on either urine spot samples or calculated assuming a zero N-balance, and excretions determined by total collection of urine only. Moreover, with the subset of data based on total collection of urine, a new dataset was created by calculating urinary N-excretion assuming zero N-balance. Comparison with the original subset of data allowed to examine the impact of such an assumption on the relationship established between milk urea-N concentration (MUN) and UN. Of all single dietary and animal factors evaluated to predict N-excretion in urine, MUN and dietary crude protein concentration (CP) were by far the best predictors. Urinary N-excretion was best predicted by the combination of MUN, CP and DMI, whereas UUN was best predicted by the combination of MUN and CP. All other factors did not, or only marginally, improve the prediction of UN or UUN. The relationship between UN and MUN differed between NA and EU with higher estimated regression coefficients for MUN for the NA dataset. Precision of UN and UUN prediction improved substantially when only UN or UUN data based on total collection of urine were used. The relationship between UN and MUN for the NA dataset, but not for the EU dataset, was substantially altered when UN was calculated assuming a zero N-balance instead of being based on total collection of urine. According to results of the present meta-analysis, UN and UUN are best predicted by the combination of MUN and CP and that, in view of precision and accuracy, prediction equations for UN and UUN should be derived from total collection of urine.

Key Words: milk urea nitrogen, urinary nitrogen, dairy cattle, meta-analysis

Introduction

Nitrogen (N) losses via excreted feces and urine in dairy cattle are associated with losses of N from the farming system through ammonia volatilization, nitrate leaching, and dissipation of N as N_2O, NO, and NO_2 (De Vries et al., 2001). In view of such environmental concerns, there is great interest in investigating the potential of specific on-farm measures to reduce N-losses, preferably without reducing milk production. Nitrogen digested and not excreted as milk protein is for the largest part excreted as urea-N in urine. On-farm indicators including milk-urea-N concentration (MUN; mg N/dL) may be attractive to monitor the excretion of urinary urea N (UUN; g N/d) or total urinary N (UN; g N/d). Several studies focused on the relationship between MUN and UN (Jonker et al., 1998; Kauffman and St-Pierre, 2001; Nousiainen et al., 2004; Zhai et al., 2005; Zhai et al., 2007). Jonker et al. (1998) and Nousiainen et al. (2004) performed meta-analyses, analyzing the relationship between MUN and UN on datasets containing data from multiple trials. Published meta-analyses have either been based solely on data from North America (NA) or data from Northwestern European countries (EU). Jonker et al. (1998) based their analysis on 3 NA trials whereas Nousiainen et al. (2004) based their analysis on a large data set of 50 EU trials with grass-silage based diets. In all trials used by Nousiainen et al. (2004) concentrates were offered at a flat rate irrespective of milk yield, and UN was not based on total collection of urine but calculated from the difference between N-intake and excretion of N in feces and milk, assuming a zero N-balance. However, it is known from various studies in lactating dairy cows (Spanghero and Kowalski, 1997; Eriksson et al., 2004; Colmenero and Broderick, 2006), mice (Costa et al., 1968), and humans (Young et al., 1981) that the N-balance in general is positive due to losses of N from the organism not measured in urine, milk, and feces. Furthermore, differences in MUN-UN relationships established in these studies might be related to differences in herd management, climatic conditions, type of diet, the concentrate : roughage ratio of the diet, the genetic makeup of the cows, or differences in techniques to measure UN and UUN. Recently, the impact of such factors has been reviewed by Spek et al. (2013a). During the last decade more attention has been paid to the relationship between MUN and UUN instead of UN because UUN is most strongly related to ammonia emission (Burgos et al., 2007). At present, only few studies (Burgos et al., 2007; Powell et al., 2011) have focused on prediction of UUN by MUN. No studies have been published on prediction of UUN from multiple animal and dietary related factors. For the present study it was furthermore hypothesized that the prediction accuracy of UN and UUN may be improved by selection of only those trials where UN and UUN are analyzed based on total collection of urine, instead of estimating UN and UUN based on the difference between N intake and excretion of N in milk and feces, or based on analysis of UN and UUN in urine spot samples with daily volume of urine estimated from creatinine levels in the same urine spot samples. For practical and animal welfare reasons it might be argued to determine UN based on the difference between N-intake and N excreted in feces and milk instead of using indwelling urine catheters. However, no studies have been carried out that have

tested whether the relationship between MUN and UN is actually similar for UN derived from total collection of urine, or for UN calculated as the difference between N-intake and N excreted in feces and milk.

The first objective of this study was to quantify the relationship between various dietary and animal factors and UN or UUN for either EU or NA datasets, and to compare their respective prediction equations. The second objective of this study was to test whether accuracy and precision of UN and UUN prediction equations are affected by the method of measuring UN and UUN, viz. estimation of UN and UUN from urine spot samples or by calculations assuming a zero N-balance, versus UN and UUN determined by total collection of urine only.

Materials and Methods

Dataset selection

Studies were selected that contained at least information on: (1) the partitioning of N-excretion in urine, feces, and milk, (2) MUN, (3) dry matter intake (DMI; kg/d) and composition of the ration, and (4) milk production and fat and protein content in milk. Mean treatment data (n = 200) from 47 trials carried out in NA (n = 118) and EU (n = 82) were used. A description of this dataset (indicated by complete dataset from hereon) is presented in Table 1 and contained 193 observations on UN (n = 111 for NA and n = 82 for EU) and 98 observations on UUN (n = 57 for NA and n = 41 for NA). The Appendix provides a reference list of 41 studies describing these 47 trials. Some studies described multiple trials and this explains the fact that there are more trials than studies. In a number of studies excretion of UN and UUN was determined based on spot samples taken from the urine, or based on calculation of UN as the difference between N-intake and excretion of N in milk and feces (i.e., zero N balance). A reduced dataset (indicated by reduced dataset) was developed that included only observations on UN or UUN from studies where urine was collected quantitatively. This reduced dataset contained 123 observations on UN (n = 55 for NA and n = 68 for EU) and 63 observations on UUN (n = 22 for NA and n = 41 for EU). The number of observations in the reduced dataset where both UN and UUN were measured was 56 (n = 15 for NA and n = 41 for EU). In order to evaluate the impact of the assumption of a zero N-balance on the results obtained, a new dataset (number of data hence identical to that of the reduced dataset) was created from the reduced dataset in which data on urine N-excretion were replaced by values calculated under assumption of a zero N-balance.

Table 1. Characterization of datasets used for the statistical evaluation.

	Northwestern Europe			North America		
	n	Mean	SD	n	Mean	SD
Animal factors						
BW (kg)	76	607	28.1	114	649	56.4
DIM	76	133	54.1	118	129	58.9
DMI (kg/d)	82	18.9	2.45	118	23.4	2.29
Milk (kg/d)	82	25.5	4.51	118	34.7	6.79
Milk urea nitrogen (mg N/dL)	82	12.5	5.07	118	13.1	3.59
Milk fat (%)	82	4.36	0.392	118	3.52	0.432
Milk protein (%)	82	3.33	0.210	118	3.05	0.262
Dietary factors[1]						
Corn silage (% in DM)	82	15	23.5	118	22	15.8
Grass silage or herbage (% in DM)	82	47	30.3	118	5	11.6
Legume (% in DM)	82	7	14.1	118	25	16.8
Roughage (% in DM)	82	68	15.3	118	52	9.4
CP (% in DM)	82	16.1	2.55	118	17.1	1.82
RDP (% in DM)	76	10.0	2.08	118	10.2	1.65
RUP (% in DM)	76	5.9	1.17	118	7.0	1.37
OEB (% in DM)	76	2.1	2.02	118	2.4	1.73
DVE (% in DM)	76	8.2	1.37	118	8.9	1.48
NE_L (Net energy lactation MJ/kg DM)	76	6.6	0.36	118	6.6	0.47
NDF (% in DM)	76	39.6	5.41	118	30.8	5.39
NDF forage (% in DM)	76	31.2	7.72	118	20.5	6.49
NDF forage:NDF total diet ratio	76	0.78	0.104	118	0.68	0.199
Starch (% in DM)	76	13.2	8.35	118	25.4	6.88
RUS (% in DM)	76	2.5	2.20	118	7.7	3.08
Ash (% in DM)	76	8.5	1.63	118	7.4	1.39
Nitrogen flows[2]						
N-intake (g N/d)	82	485	85.0	118	637	89.6
N-milk (g N/d)	82	133	21.0	118	166	32.1
N-feces (g N/d)	82	159	31.9	111	223	62.9
UN (g N/d)	82	185	69.1	111	212	56.0
UUN (g N/d)	41	152	85.7	57	168	47.9
NUUN (g N/d)	35	45	12.0	50	44	19.5
N balance (g N/d)	68	10	30.9	55	54	38.8

[1]CP = crude protein, RDP = rumen degradable protein, RUP = rumen undegradable protein, OEB = rumen-degraded protein balance (Tamminga et al., 1994), DVE = intestinal digestible protein (Tamminga et al., 1994), NE_L = Net energy for lactation (Van Es, 1975), RUS = rumen undegradable starch.

[2]UN = N excreted in urine, UUN = urea-N excreted in urine, and NUUN = non-urea-N excreted in urine. N-balance = calculated as N-intake minus N excreted in urine, feces, and milk where UN is based on total collection of urine.

Independent and dependent factors

The list of independent factors that were tested for their capacity to explain observed variation in UN and UUN included animal factors and dietary factors. These independent factors are presented in Table 1 under the headings 'Animal factors' and 'Dietary factors'. The dependent factors in the dataset were UUN and UN. There were some missing dietary values with respect to ash, starch, and NDF. These missing values were predicted based on typical composition using the Dutch feeding tables (CVB, 2007). For all diets, values were predicted for rumen degradable protein (RDP), rumen undegradable protein (RUP), rumen undegradable starch (RUS), digestible protein available in the small intesting (DVE), rumen degradable protein balance (OEB), and the net energy content of the diet (NE_L) using the Dutch feeding tables (CVB, 2007).

Statistical procedure

Multiple regression analyses were carried out with the MIXED procedure in SAS with trial included as random effect and all other factors as fixed effects. Fixed effects were nested within continent (EU or NA). Univariate regression analysis of fixed factors on UN was carried out to determine those factors that explained most variation in UN from the complete dataset. Those factors that explained more than 50% of variation in UN were combined with each of the other individual animal and dietary related factors by means of multivariate regression analysis. Based on the criteria of R^2, root mean square prediction error (RMSPE), and Akaike's information criterion (AIC) it was decided whether the other factors improved the model fit. It was assumed that observations within trial were equally correlated to each other and therefore the covariance structure was modeled as compound symmetry. The R^2 values presented apply to the regression of predicted values of fixed effects to observed values, hence excluding the contribution of variation associated with random effect. Likewise, the RMSPE was calculated from the residuals of the fixed effects. The RMSPE was calculated according to Jonker et al. (1998). In practice, the trial effect is unknown and was not included in the calculation of RMSPE and R^2, and therefore better reflects the prediction accuracy to be expected when applying the models. A COVTEST statement was included in the PROC MIXED statement to test for the effects of fixed factors on changes in between-trial variation. Data from one of the trials of Tas et al. (2006) were discarded because of very low MUN values that resulted in UN:MUN ratios more than three standard deviations higher than the average.

Results and Discussion

Dataset evaluation

The complete dataset showed a large range in CP (9.4 – 24.1 % DM), MUN (3.8 - 30.2 mg N/dL), DMI (13.8 – 30.2 kg/d) and milk production (15.5 – 45.5 kg/d). The NA diets were largely based on corn silage and alfalfa and contained on average 52% roughage, whereas the

EU diets were largely based on grass silage and clover silage and contained on average 68% roughage. As a consequence, the NA formulated rations contained on average 25.4 % starch (DM basis) and EU rations contained 13.2% starch (DM basis). On average, NA cows produced 9.2 kg/d more milk (34.7 vs. 25.5 kg/d), consumed 4.5 kg DM/d more feed (23.4 vs. 18.9 kg DM/d), and were 42 kg heavier (649 vs. 607 kg) than the EU cows. Higher standard deviations were found in the EU studies compared to NA studies for MUN (SD of 5.07 vs. 3.59), UN (SD of 69.1 vs. 56.0) and UUN (SD of 85.7 vs. 47.9). Daily N-intake was on average 152 g higher in NA studies and daily excretion of N in milk, urine, and feces were 33, 27, and 64 g higher, respectively. Average MUN was 0.6 mg/dL higher in NA compared to EU, in line with the higher UN and UUN, but lower than would be expected considering the 27 (UN) and 16 (UUN) g N/d difference between EU and NA. Compared to EU data, fecal N-excretion was 40% higher for NA cows. whereas DMI and CP content for NA were only 24% and 6% higher, respectively. The positive relation between fecal N-excretion and DMI might explain the higher fecal N-excretion for NA. Huhtanen et al. (2008) observed a 9.9 g increase in fecal N per kg increase of DMI. Regression of fecal N-excretion on DMI in this study resulted in 11.3±1.07 and 12.1±2.03 g fecal N per kg DMI for EU and NA, respectively. The intercept was 17.4±48.77 g fecal N/d higher for NA and not statistically different from EU. Hristov et al. (2005) found a 76% higher fecal N-excretion for diets supplemented with starch compared to diets supplemented with fiber and ascribed this difference in fecal N to differences in amounts of microbial N synthesized in the large intestine and excreted in the feces. The 17.4 g higher fecal N-excretion for NA, not being explained by DMI, might be explained by the larger amounts of RUS reaching the large intestine for NA diets, resulting in an increased synthesis and fecal excretion of microbial N.

Explanatory factors

From all independent factors tested, the animal factor MUN and the dietary factor CP as the single explanatory factor in the model explained most of the observed variation of UN (R^2 of 0.72 and 0.79, respectively; Table 3) and of UUN (R^2 of 0.87 and 0.81, respectively; Table 4). Combining MUN and CP further improved the explanation of observed variation of UN and UUN (Tables 3 and 4). Although a substantial portion of variation in UN in the complete dataset was explained by the single factors RUP (38%), RDP (36%), OEB (37%), and DVE (29%) (Table 2), these factors did not increase the portion of explained variation when added to the model that already included MUN and CP. For UUN, a substantial part of variation in UUN could be explained by the single factors RDP (50%), OEB (49%), DVE (20%), and RUP (18%) but, similarly to UN, these factors did not increase the portion of explained variation when added to the model that already included MUN and CP. The prediction of UN was only slightly further improved when DMI was added to the model that already included MUN and CP. Milk production and BW were significant factors in explaining variation in UN when added to the model that already included MUN and CP. However, the model fit as

judged by the RMSPE and R^2, did not improve and therefore, these factors were excluded from the models presented in Tables 3 and 4.

Table 2. Effect of single factors on N-excretion in urine in the complete dataset as judged from the P-value of these single factors and the goodness of fit parameters R^2 and AIC.

	P-value	R^2[1]	AIC[2]
Animal factors			
MUN (mg N/dL)	<0.001	0.72	1,683
BW (kg)	0.050	0.11	1,932
DIM	0.480	0.07	1,979
Milk (kg/d)	0.010	0.01	2,025
Milk protein (%)	0.449	0.07	2,019
Milk fat (%)	0.350	0.05	2,021
DMI (kg/d)	0.008	0.02	2,021
Dietary factors			
CP (% in DM)	<0.001	0.79	1,700
RDP (% in DM)	<0.001	0.36	1,750
RUP (% in DM)	<0.001	0.38	1,903
OEB (% in DM)	<0.001	0.37	1,779
DVE (% in DM)	<0.001	0.29	1,925
NE_L (MJ/kg DM)	0.075	0.12	1,955
NDF (% in DM)	0.518	0.01	1,970
NDF forage (% in DM)	0.974	0.06	1,972
Starch (% in DM)	<0.001	0.28	1,943
RUS (% in DM)	<0.001	0.21	1,950
Ash (% in DM)	0.004	0.14	1,955
Roughage (% in DM)	0.814	0.10	2,032

[1]R^2 is calculated from the total sums of squares of the model and the sums of squares explained by the fixed effect part of the model only.

[2]AIC = Akaike information criterion. A measure of the relative goodness of fit of the model. A smaller value means a better model fit.

Impact of dietary and animal factors on UN

More variation of UN in the complete dataset was explained by CP (R^2 = 0.79) than by MUN (R^2 = 0.72), whereas in the reduced dataset MUN explained slightly more variation than CP (R^2 = 0.85 vs. 0.82). However, both the complete and reduced datasets had lower AIC values when MUN was included in the model, thus favoring MUN above CP as a predictor of UN. Results from a meta-analysis by Nousiainen et al. (2004) suggest that, based on R^2 and root mean square errors values, CP is a better predictor of UN than MUN (R^2 of 0.85 vs. 0.74). However, Nousiainen et al. (2004) did not report AIC values and therefore, based on the combined results of the present study and the study of Nousiainen et al. (2004), it is not

possible to conclude which predictor, CP or MUN, is preferred to predict UN. The relationship established between MUN and UN was almost identical for the complete and reduced dataset, for EU as well as for NA (Fig. 1, Table 3). Results of univariate regression of UN on MUN for NA and EU were similar to values reported by Kauffman and St-Pierre (2001), Kohn et al. (2002), Nousiainen et al. (2004), and Zhai et al. (2005) at low to moderate MUN values (MUN<10 mg N/dL). Predicted values of UN from the study of Kauffman and St-Pierre (2001), Kohn et al. (2002), Nousiainen et al. (2004), and predicted values of NA in the present study diverged (became larger) at higher MUN values from predicted values from the study of Zhai et al. (2005) and predicted values of EU in the present study (Fig. 1).

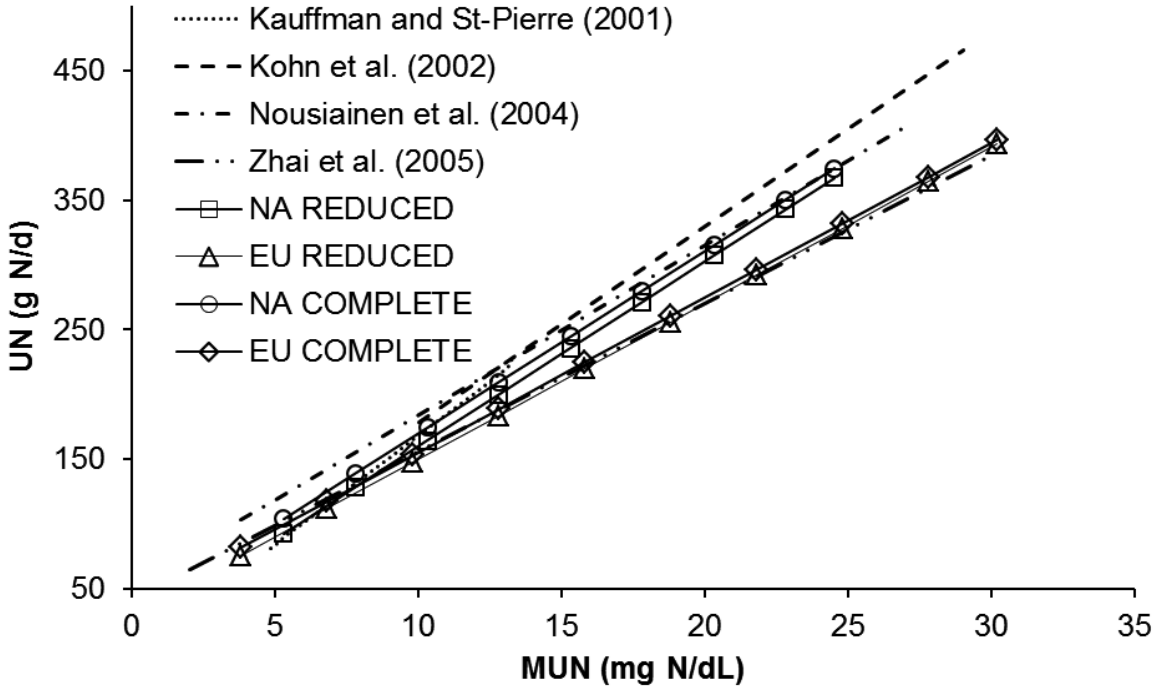

Figure 1. Relationship between milk urea nitrogen (MUN; mg N/dL) and urinary nitrogen excretion (UN; g N/d) for Northwestern Europe (EU) and North America (NA) in case UN is determined based on the complete dataset (COMPLETE; n=193) or based on the reduced dataset (REDUCED; n=123) in this study, and for other studies (Kauffman and St-Pierre, 2001; Kohn et al., 2002; Nousiainen et al., 2004; Zhai et al., 2005).

Multiple regression of MUN and CP on UN improved the explanation of UN substantially as judged by the AIC, R^2, and RMSPE for both the complete (model 3; Table 3), reduced (model 7; Table 3), NA, and EU datasets. Furthermore, this model lowered the differences in regression coefficients for MUN, but increased the differences in regression coefficients for CP between EU and NA. Besides the increase in differences between regression coefficients for CP for EU and NA, the differences in intercepts between NA and EU increased as well,

from 10.2 to 65.8 g N/d for the complete dataset and from 15.7 to 58.0 g N/d for the reduced dataset, respectively, indicating an interaction between CP and MUN. Indeed, a positive significant interaction between MUN and CP was observed for the complete dataset (P=0.006) and reduced dataset (P=0.006). Apparently, CP and MUN are not completely confounded in explaining variation in UN as can be observed from the increased explanation of variation in UN when both factors are included in the model compared to univariate models containing either MUN or CP. The interaction between CP and MUN is another indication that CP and MUN are not completely confounded with respect to explaining variation in UN. Nousiainen et al. (2004) observed a positive linear relationship between MUN and CP; MUN (mg N/dL) = 1.7 × CP (% in DM) – 14.2 with an R^2 of 0.78. In the present study, a similar relationship was established between CP and MUN for EU ($R^2 = 0.75$) but a weaker one for NA ($R^2 = 0.47$) (Fig. 2).

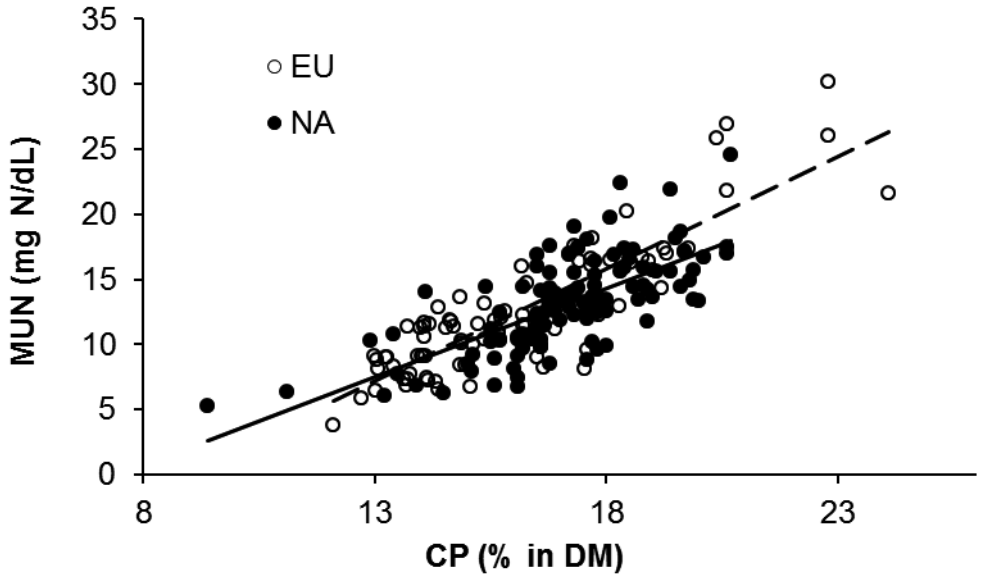

Figure 2. Relationship between dietary crude protein content (CP: % in DM) and milk urea nitrogen (MUN; mg N/dL) for Northwestern Europe (EU; n = 82) and North America (NA; n = 118) based on the complete dataset. For NA (solid regression line); MUN (mg N/dL) = -10.1±2.29 + 1.36±0.133 × CP (% in DM), R^2 = 0.47. For EU (dashed regression line); MUN (mg N/dL) = -15.3±1.80 + 1.73±0.111 × CP (% in DM), $R^2 = 0.75$.

The fact that a substantial part of variation in MUN is not explained by CP indicates that at a given level of CP the level of MUN varies due to other factors than CP. Factors that might explain this unexplained variation in MUN are numerous and are related to differences in nutrition and management, and differences between cows. For instance, nutritional factors that affect MUN, but that are unrelated to CP, include the partition of CP in RDP and RUP (Broderick et al., 1993), the dietary intake of salt and water (Burgos et al., 2001; Spek et al.,

2012b), or dietary energy content (Broderick and Clayton, 1997). Examples of management factors that affect MUN, but not CP, are the time and frequency of feeding and milking, which can both affect the diurnal pattern of MUN (Gustafsson and Palmquist, 1993; Friggens and Rasmussen, 2001). Differences between cows with respect to milk protein production might affect the partition of degraded and absorbed protein into milk protein and urea formed with protein catabolism, thereby affecting the relationship between MUN and CP (Huhtanen and Hristov, 2010). Furthermore, the need of the animal to retain N at low levels of N-intake affects the renal regulation of urea excretion. Several studies in sheep (Schmidt-Nielsen et al., 1958), goats (Eriksson and Valtonen, 1982), and cattle (Thornton, 1970) show that a reduction in CP can result in a reduced renal urea clearance rate and an increased renal urea reabsorption. These kidney related factors affect the pool of urea and the distribution of urea in the cow, and as a result affect concentrations of urea nitrogen in blood plasma and MUN. All sources of variation mentioned above might underlie the moderate relationship observed between MUN and CP. The same sources of variation may be responsible for the added effect of MUN and CP in predicting UN. In the present study there are also indications for an increase in renal recycling of urea at low dietary CP as observed from differences in quantities of urinary urea-N excreted per unit increase in MUN at low and high protein diets. For example, regression of UUN on MUN for the reduced dataset resulted in lower regression coefficients for MUN for EU (8.01±2.683) for a sub-dataset with only CP values lower than average CP, compared to regression coefficients for EU (13.70±0.759) for a sub-dataset with CP values higher than average CP. These differences in regression coefficients of MUN between high and low protein sub-datasets in predicting UUN are also reflected by the positive interaction between CP and MUN in the complete (P=0.006) and reduced (P=0.006) dataset in predicting UN, even though inclusion of the CP × MUN interaction did not improve model fit. These differences in regression coefficients for MUN found in the sub-dataset with low CP values and those observed in the sub-dataset with high CP values might explain differences in UN-MUN relationships found between various studies, and might also explain differences in UN-MUN relationships observed in the present dataset between NA and EU data.

Addition of DMI to the model containing MUN and CP (models 4 and 8; Table 3) slightly improved the model fit of UN without a pronounced effect on regression coefficients of MUN and CP (Table 3). However, the difference in intercept values between EU and NA was reduced. The effect of DMI on UN was larger for EU than NA and is likely related to the positive and significant relation between CP and DMI for NA in the complete (P=0.044) and reduced (P=0.040) dataset whereas no such relationship was established for EU in the complete (P=0.530) and reduced (P=0.590) dataset. The authors of the present study have no explanation for these differences in CP - DMI relationships between NA and EU.

Impact of dietary and animal factors on UUN

Variation in UUN was largely explained by MUN (models 9 and 12; Table 4) and CP (models 10 and 13; Table 4) with the complete as well as the reduced dataset. Milk urea nitrogen explained a larger fraction (R^2=0.87 – 0.93) of observed variation in UUN than CP (R^2=0.81), with 93% of variation in UUN explained for the EU data and 75% of NA data. The fact that more variation of UUN was explained in EU compared to NA data may be related to the larger variation in the EU data vs. the NA data for UUN (152±85.7 vs. 168±47.9 g N/d) and MUN (12.5±5.07 vs. 13.1±3.59 mg N/dL). The slope of MUN on UUN was similar for EU (14.09 and 14.15 for the complete and reduced dataset, respectively) and NA (14.40 and 14.57 for the complete and reduced dataset, respectively) whereas the intercept value of UUN tended to be lower for the EU than the NA dataset for both the complete and reduced dataset. Burgos et al. (2007) and Powell et al. (2011) observed regression coefficients for MUN of 14.4 and 16.2 g urinary urea N/d per unit of MUN, respectively. The model prediction of Burgos et al. (2007) was based on a single study, whereas the model prediction of Powell et al. (2011) was based on simple regression of UUN on MUN data from 9 studies without correction for trial effect. The reason that a higher regression coefficient for MUN was found by Powell et al. (2011) compared to Burgos et al. (2007) and results from the present study might be the consequence of not accounting for trial effect as shown by St-Pierre (2001). Extrapolation of the model prediction of UUN by CP (model 13; Table 4) suggests that UUN would become zero at CP of 12.8 and 11.3% with the NA and the EU data, respectively, suggesting that these CP values indicate the highest possible N-utilization with no loss of urea excreted in urine. Few studies have determined minimal UN or UUN excretion in dairy cows. In one study, Wohlt et al. (1978) measured a UN of 35 to 38 g N/d at CP of 9.4 to 10.3 %, a UN of 57 to 63 g N/d at CP of 11.6 to 12.0 %, and a UN of 115 to 137 g N/d at CP of 14.2 to 14.4 %. In another study, Ørskov and MacLeod (1982) measured minimal UN and UUN in pregnant non lactating Friesian cows receiving no dietary N at all and observed UN ranging from 34.2 to 42.0 g N/d and UUN ranging from 17.8 to 26.0 g N/d. The similarity in UN values observed in the study of Ørskov and MacLeod (1982) and those observed in the study of Wohlt et al. (1978) at CP ranging from 9.4 to 12.0 % indicate that the cow is able to reduce the excretion of UN to 34 – 42 g N/d. A third study, a review on urea excretion and recycling to the gastrointestinal tract (Reynolds and Kristensen, 2008) demonstrated that ruminants are able to reduce urea excretion at low CP, and observed that at CP of 7.5% the amount of urea excreted as a proportion of urea produced became close to zero. The database used in the present study indicates an average non-urea urinary nitrogen excretion (NUUN; g N/d) of 45±12.0 and 44±19.5 g N/d for the EU and NA dataset, respectively (Table 1). It was expected that regression coefficients for MUN and CP vs. UUN would be similar to those obtained in the UN equation, as UUN and UN are strongly related (Burgos et al., 2005; and Fig. 3 showing results of the present study). This was indeed the case for the NA dataset where MUN regression coefficients of UN models for both the complete (14.08) and reduced

(14.31) datasets (Table 3), were similar to the MUN regression coefficients (14.40 and 14.57, respectively) of the UUN models (Table 4). However, for the EU dataset MUN regression coefficients of the UN models for the complete (11.92) and reduced (12.03) datasets (Table 3), were lower than the MUN regression coefficients (14.09 and 14.15, respectively) of the UUN models (Table 4). Removal of extreme MUN observations (>25 mg N/d) from the EU dataset reduced the MUN regression coefficients for UUN from 14.15 to 12.71 in the reduced dataset and from 14.09 to 12.62 in the complete dataset, and became similar to those obtained for UN. The observations with MUN values higher than 25 mg N/dl in the EU dataset were therefore probably responsible for this effect. Regression coefficients of CP were higher in the UUN models for the complete and reduced dataset (models 10 and 13; Table 4) compared to those for the UN models, in particular for the NA dataset.

Similar to the prediction of UN, the combination of MUN and CP in both the complete and reduced dataset (models 11 and 14, respectively; Table 4) resulted in a better prediction of UUN than with MUN or CP as the single explanatory factor, lowering the AIC and RMSPE substantially. The same physiological explanation as given for the added effect of MUN and CP in explaining UN holds for UUN. Addition of other dietary or animal factors did not improve the explanation of UUN.

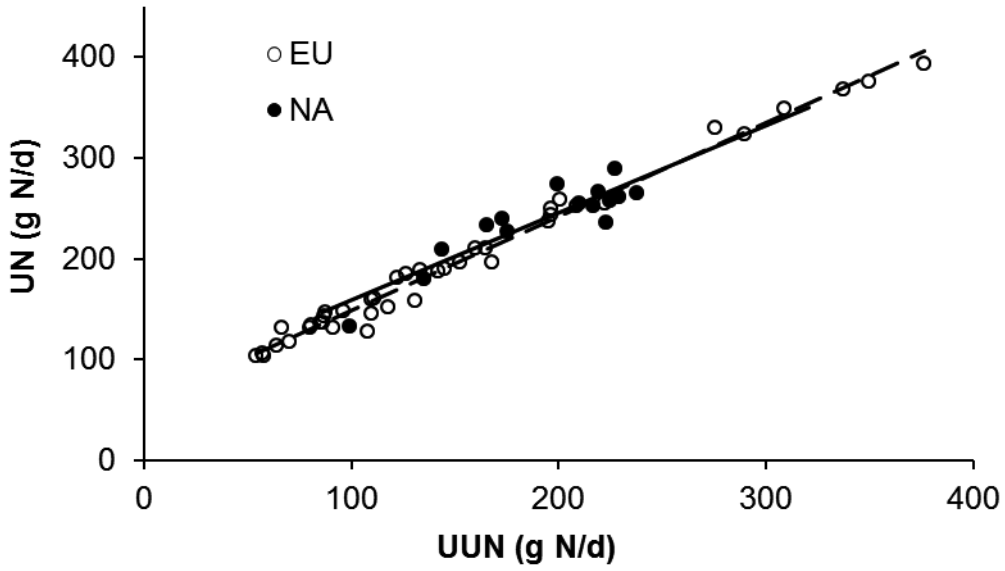

Figure 3. Relationship between urinary urea nitrogen excretion (UUN; g N/d) and urinary total nitrogen excretion (UN; g N/d) for Northwestern Europe (EU; n = 41) and North America (NA; n = 15) based on the reduced dataset. For NA (solid regression line); UN (g N/d) = 72.3±22.49 + 0.866±0.1147 × UUN (g N/d), R^2 = 0.81. For EU (dashed regression line); UN (g N/d) = 55.4±3.14 + 0.932±0.0181 × UUN (g N/d), R^2 = 0.99.

Between-trial variation

Between-trial variation for UN in a model containing only the class variable continent (NA or EU) was substantially reduced when MUN (reduction of 63 and 80% for the complete and reduced dataset, respectively), CP (reduction of 77 and 80% for the complete and reduced dataset, respectively), or the combination of MUN and CP (reduction of 82 and 93% for the complete and reduced dataset, respectively) was included in the model. Between-trial variation for UUN in a model containing only the class variable continent (NA or EU) was substantially reduced when MUN (reduction of 78 and 91% for the complete and reduced dataset, respectively), CP (reduction of 73 and 70% for the complete and reduced dataset, respectively), or the combination of MUN and CP (reduction of 87 and 97% for the complete and reduced dataset, respectively) was included in the model. Trial effect was significant in all cases except for the multivariate model containing MUN and CP that predicted UUN for the reduced dataset (P=0.066). The reduction in between-trial variation upon including MUN, CP, or the combination of MUN and CP in the model is not surprising and can be explained by the variation in UN and UUN explained by MUN and CP. The significance of trial effect in almost all cases indicates that there is still additional explainable variation present.

Table 3. Prediction of urinary nitrogen excretion (UN; g N/d) by means of milk urea nitrogen concentration (MUN; mg N/dL), dietary CP concentration (CP; % in DM), and dry matter intake (DMI; kg/d). Models 1 – 4 are based on studies where UN was determined based on; total collection of urine, urine spot samples, or zero N-balance calculations (dataset COMPLETE) whereas models 5 – 8 are based on studies where UN was determined based on total collection of urine only (dataset REDUCED).

Model	Dataset	μ[1] SE P-value	EU[2] SE P-value	MUN SE P-value		CP SE P-value		DMI SE P-value		n[4]	R²[5]	RMSPE[6]	AIC[7]
				EU	NA[3]	EU	NA	EU	NA				
		29.3	7.1	11.92**	14.08								
1	COMPLETE	10.38	14.41	0.470	0.657					193	0.72	33.1	1,683
		0.007	0.621	<.001	<.001								
		-223.9	10.2			24.81	25.47						
2	COMPLETE	22.61	29.53			1.089	1.294			193	0.79	28.6	1,700
		<.001	0.730			<.001	<.001						
		-132.2	65.8	7.73	7.52	9.63	14.42						
3	COMPLETE	21.46	33.39	1.086	0.962	2.294	1.745			193	0.85	24.7	1,609
		<.001	0.051	<.001	<.001	<.001	<.001						
		-168.9	19.1	7.55	7.84	9.64	13.37	4.55	2.17				
4	COMPLETE	29.43	41.10	1.015	0.9100	2.143	1.700	0.895	1.182	193	0.86	24.2	1,578
		<.001	0.646	<.001	<.001	<.001	<.001	<.001	0.069				
		16.7	13.0	12.03*	14.31								
5	REDUCED	12.94	15.82	0.466	0.824					123	0.85	26.4	1,050
		0.206	0.413	<.001	<.001								
		-230.5	15.7			24.65	25.72						
6	REDUCED	32.98	39.16			1.219	1.880			123	0.82	28.6	1,094
		<.001	0.690			<.001	<.001						
		-123.8	58.0	8.27	8.95	8.87	12.43						
7	REDUCED	27.23	36.91	1.077	1.235	2.256	2.237			123	0.93	18.8	1,012
		<.001	0.120	<.001	<.001	<.001	<.001						
		-148.8	8.9	8.06	8.79	8.91	11.97	4.06	1.58				
8	REDUCED	39.79	49.42	1.019	1.213	2.132	2.178	0.915	1.583	123	0.93	18.3	988
		<.001	0.858	<.001	<.001	<.001	<.001	<.001	0.322				

[1,2,3,4,5,6,7]A description of these footnotes is provided in the footnote of Table 4.

Table 4. Prediction of urinary urea nitrogen excretion (UUN; g N/d) by means of milk urea nitrogen concentration (MUN; mg N/dL) and dietary CP concentration (CP; % in DM). Models 9 – 11 are based on studies where UUN was determined based on; total collection of urine, urine spot samples, zero N-balance calculations (dataset COMPLETE) whereas models 12 – 14 are based on studies where UUN was determined based on total collection of urine only (dataset REDUCED).

Model	Dataset	μ[1] SE P-value	EU[2] SE P-value	MUN SE P-value EU	MUN SE P-value NA[3]	CP SE P-value EU	CP SE P-value NA	n[4]	R^2[5]	RMSPE[6]	AIC[7]
9	COMPLETE	-20.8 11.05 0.075	-26.6 15.74 0.095	14.09 0.535 <.001	14.40 0.701 <.001			98	0.87	23.7	825
10	COMPLETE	-380.7 38.03 <.001	56.2 47.03 0.236			28.70 1.523 <.001	32.00 2.182 <.001	98	0.81	29.0	874
11	COMPLETE	-197.6 35.78 <.001	74.3 46.23 0.112	10.97 1.248 <.001	8.77 1.231 <.001	7.19 2.645 0.008	14.62 2.829 <.001	98	0.92	18.9	788
12	REDUCED	-31.4 16.21 0.077	-17.2 19.14 0.374	14.15 0.541 <.001	14.57 0.93 <.001			63	0.93	19.9	523
13	REDUCED	-470.4 61.95 <.001	144.6 69.07 0.042			28.77* 1.657 <.001	36.82 3.355 <.001	63	0.81	33.6	564
14	REDUCED	-146.9 62.82 0.038	2.7 67.87 0.968	10.38 1.119 <.001	11.32 1.839 <.001	8.92 2.315 0.004	9.19 4.771 0.060	63	0.96	15.5	500

[1]μ = intercept value. [2]EU = data in the column EU are valid for Northwestern Europe only and this factor is included in the model to be able to create separate intercepts and regression coefficients for North America and Europe. [3]NA = data in the column NA are valid for North America only and this factor is included in the model to be able to create separate regression coefficient for North America and Northwestern Europe. [4]n = Number of observations. [5]R^2 is calculated from the total sums of squares of the model and the sums of squares explained by the fixed effect part of the model only. [6]RMSPE = Root mean square prediction error and based on the residuals from the fixed effect part of the model only. [7]AIC = Akaike information criterion. A measure of the relative goodness of fit of the model. A smaller value means a better model fit. *P≤0.05, **P≤0.01, ***P≤0.001. Asterisks denote a significance of difference in regression coefficients for MUN, CP, or DMI between EU and NA.

Precision and accuracy

A more precise explanation of UN and UUN was obtained with the reduced dataset, containing only observations on UN and UUN based on total collection of urine, compared to the complete dataset, as judged by the R^2 and RMSPE. The RMSPE obtained for UN using both MUN and CP as independent variables in the model was reduced by 24% (from 24.7 to 18.8; model 3 and 7 in Table 3). The RMSPE obtained for UN using MUN, CP, and DMI as independent variables in the model was reduced by 24% as well (from 24.2 to 18.3; model 4 and 8 in Table 3). The RMSPE for the UUN dataset using both MUN and CP as independent variables in the model was reduced by 18% (from 18.9 to 15.5; model 11 and 14 in Table 4). These substantial reductions in RMSPE for the reduced dataset (vs. complete dataset) demonstrates the importance of analyzing UN and UUN based on total collection of urine instead of determining UN and UUN from urine spot samples or by assuming zero N-balance. Although for the reason of precision it is best to estimate UN based on total collection of urine (UN-analyzed), from a practical and animal welfare point of view it might be preferred to derive UN by assuming a zero N-balance and calculate UN as the difference between N-intake and N excreted in feces and milk (UN-calculated). Although precision of UN-calculated will be lower, little is known about the effect of calculating UN based on zero N-balance on accuracy. The MUN - UN-calculated relationship and the MUN - UN-analyzed relationship for the same reduced dataset were compared and it appeared that these relationships were similar for EU but substantially different for NA (Fig. 4). For the EU dataset, the combined intercept and the slope were 29.7 and 12.03±0.466 for the analyzed data, and 37.0 and 12.33±0.764 for the calculated data. The relationship between UN and MUN for the NA dataset had a higher regression coefficient for MUN when UN was calculated (17.46±1.352; Fig. 4) as compared to UN measured (14.31±0.824; model 5 in Table 3). These results indicate that the accuracy of MUN in predicting UN is sensitive to the method of UN determination. The higher UN-calculated, compared to UN-analyzed, can only be explained by a high positive N-balance for NA. The reduced dataset average N-balance was 54±38.8 g N/d for NA and substantially larger than the average N-balance for EU of 10.1±30.9 g N/d (Table 1). It is unclear why the N-balance is higher for the NA than for the EU dataset. Spanghero and Kowalski (1997) observed from a meta-analysis on N-balance trials (35 studies, 27 from NA and 8 from EU) an average positive N-balance of 39 g N/d which seems in line with the findings in the present study for the NA dataset. Spanghero and Kowalski (1997) also observed a positive relationship between digestible N-intake and N-balance. This positive relationship might also explain the difference in N-balance between EU and NA as N-intake (and probably also digestible N-intake) was on average 31% higher for NA (637 g N/d) compared to EU (485 g N/d).

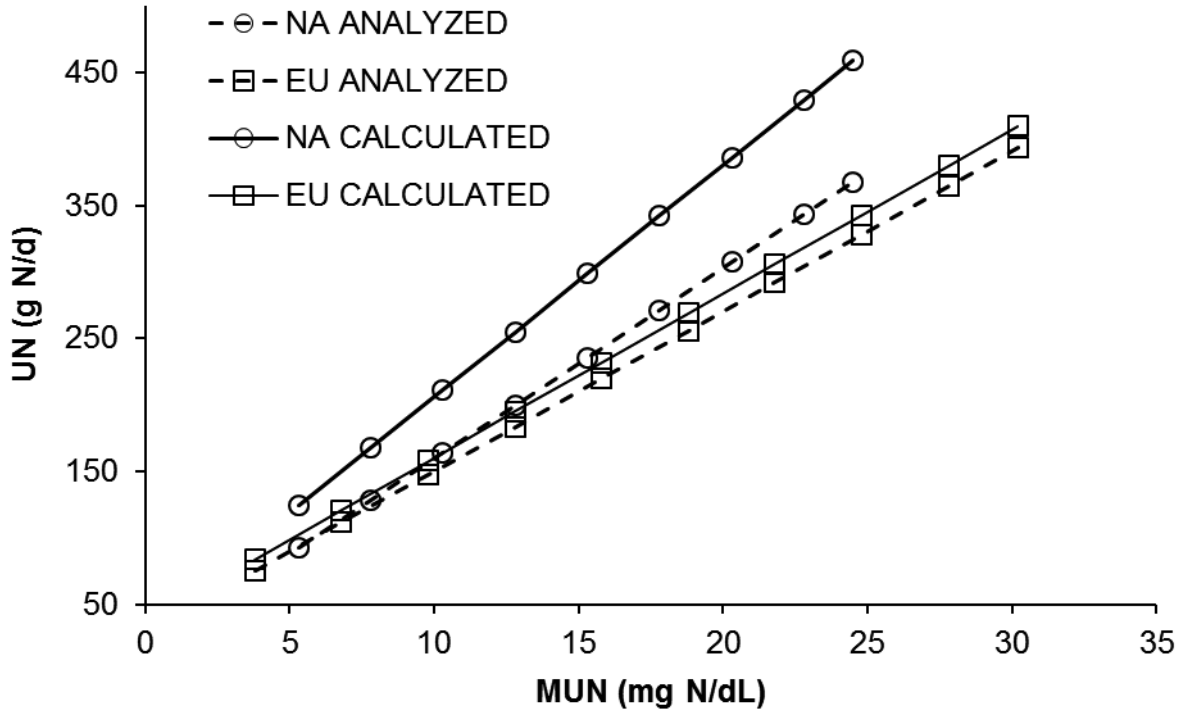

Figure 4. Relationship between milk urea nitrogen (MUN; mg N/dL) and urinary nitrogen excretion (UN; g N/d) based on data from the reduced dataset for Northwestern Europe (EU; n = 68) and North America (NA; n = 55) in case UN is determined based on total collection of urine (ANALYZED) or based on calculation of UN as the difference between N-intake and N excreted in feces and milk (CALCULATED). The UN ANALYZED prediction model is presented in model 5 (Table 3). UN CALCULATED prediction model; μ = 31.4±21.22, EU = 5.6±25.94, EU regression coefficient for MUN = 12.33±0.764, NA regression coefficient for MUN = 17.46±1.352, AIC = 1,168, RMSPE = 43.8, R^2 = 0.79.

Conclusions

Variation in UN and UUN for EU and NA was best explained by the combination of MUN and CP. Addition of DMI to the model further improved the explanation of UN for EU, but not for NA. Other animal and dietary factors tested in this study did not, or only marginally, improve the model fit of UN and UUN. The relationship between UN and MUN differed between NA and EU with higher estimated regression coefficients for MUN for the NA dataset. Prediction precision of UN and UUN was improved substantially by including only those studies in which UN and UUN were based on total collection of urine instead of being derived from spot samples or by assumption of zero N-balance. In view of precision and accuracy, relations between MUN and UN should be based on UN derived from total collection of urine whereas establishing relationships between MUN and UN where UN is calculated as N-intake minus N excreted in milk and feces is likely to yield inaccurate and imprecise relationships. It is concluded that on-farm prediction of UN or UUN can be

substantially improved by using MUN and CP compared to either MUN or CP alone and might help in monitoring and in taking policy measures to reduce environmental N-losses.

Acknowledgements

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

Appendix

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Chapter 4

Effect of sodium chloride intake on urine volume, urinary urea excretion, and milk urea concentration in lactating dairy cattle

J.W. Spek[1,2], A. Bannink[2], G. Gort[3], W.H. Hendriks[1], and J. Dijkstra[1],

[1]Animal Nutrition Group, Wageningen University, PO Box 338, 6700 AH, Wageningen, The Netherlands
[2]Department of Animal Nutrition, Wageningen UR Livestock Research, PO Box 65, 8200 AB, Lelystad, The Netherlands
[2]Biometris, Wageningen University, PO Box 100, 6700 AC, Wageningen, The Netherlands

Journal of Dairy Science (2012) 95, 7288-7298

Abstract

Milk urea nitrogen (MUN; mg N/dL) has been shown to be related to urinary excretion of urea-N (UUN; g N/d) and total urinary excretion of N (UN; g N/d) in dairy cows. In the present experiment, it was hypothesized that MUN and the relationship between MUN and UUN or UN is affected by urine volume as a result of dietary sodium chloride intake. Twelve lactating Holstein Friesian dairy cows (milk production 28.1±3.23 kg/d and 190±41 DIM), of which four were fitted with catheters in the urine bladder and jugular vein, were randomly assigned to four dietary levels of sodium chloride (3, 9, 14, and 19 g Na/kg DM) according to a triple 4 × 4 Latin square design. Cows were fed at 95% of ad libitum excluding salt addition. Milk was analyzed for MUN and protein content, urine was analyzed for total N, urea, and creatinine, feces was analyzed for total N and DM, and blood plasma was analyzed for urea and creatinine. Creatinine clearance rate (CCR; L/min) and renal urea reabsorption ratio were estimated based on plasma concentrations of urea and creatinine, and total excretion of urea and creatinine in urine. Intake of DM and N, milk production, and milk protein content were on average 21.4±1.24 kg/d, 522±32.0 g/d, 25.4±2.53 kg/d, and 3.64±0.186%, respectively. A linear relationship was found between Na intake and urine production (urine (kg/d) = 7.5±4.33 + 0.136±0.0143 × Na intake (g/d)) and between Na intake and MUN (MUN (mg/dL) = 13.5±0.35 – 0.0068±0.00104 × Na intake (g/d)). Despite the decrease in MUN with increased Na intake, UN excretion increased linearly with Na intake. Excretion of UUN was not affected by dietary Na content. A linear plateau relationship was observed between CCR and renal urea reabsorption. An increase in CCR coincided with an increase of calculated renal urea reabsorption until a CCR breakpoint value of 1.56±0.063 L/min was reached. It is concluded that Na intake is negatively related to MUN whereas UUN is not affected. Variation in mineral intake levels that affect urine volume should therefore be taken into account when using MUN as an indicator of UUN in dairy cattle.

Key Words: milk urea nitrogen, urinary urea nitrogen excretion, salt intake, urine production

Introduction

There are concerns with the negative effects of ammonia emission to the environment (Draaijers et al., 1989; Howarth et al., 1996). Livestock, in particular cattle, are responsible for the majority of ammonia emission (Pain et al., 1998; Hutchings et al., 2001). In dairy cows, the primary source of ammonia-N from manure is urinary urea-N (UUN; g N/d), which is hydrolyzed to ammonia and carbon dioxide by the activity of microbial urease present in the feces. An increase in plasma urea nitrogen concentration (PUN; mg N/dL) leads to elevated concentrations of milk urea-N (MUN; mg N/dL) and increased excretion of UUN (Burgos et al., 2007). There is a positive correlation between MUN and UUN which has led to the development of several predictive models to estimate UUN based on MUN (Burgos et al., 2007; Powell et al., 2011). In addition, models have been developed that predict urinary N-excretion (UN; g N/d) based on MUN alone (Jonker et al., 1998) or with additional parameters included such as body weight (Kauffman and St-Pierre, 2001) or daily milk production (Nousiainen et al., 2004). The robustness of these relationships has been challenged however. Bannink and Hindle (2003) using individual cow data from Dutch N-balance trials showed that a significant proportion of the variation (28%) in UN could not be explained by the factor MUN in a wide MUN range of 5 to 30 mg N/dL. Using a limited MUN range of the dataset (5 to 15 mg N/dL) to represent the relevant range in practice, Bannink and Hindle (2003) showed that the accuracy of predicting UN from MUN declined with 77% of the variation remaining unexplained.

Under a wide range of dietary conditions, dietary protein content explains most of the variation in MUN (Nousiainen et al., 2004). However, there are a number of (interrelated) factors, other than dietary protein content, that affect MUN (Spek et al., 2013a). One of these factors is the volume of urine produced. A number of studies show that water intake has an effect on PUN (Weeth and Haverland, 1961; Weeth and Lesperance, 1965; Utley et al., 1970; Godwin and Williams, 1984) and on MUN (Burgos et al., 2001). The effect of water intake, or urine production, on PUN and MUN might affect the relationship between MUN and UN or UUN. The main objective of this study was to quantify the effect of dietary Na-intake on urine production level, PUN, MUN, UN, and UUN, and on the relationship between MUN and UN or UUN. The second objective of this study was to study the effect of dietary Na-intake on the volume of blood filtrated by the kidneys as represented by the creatinine clearance rate (CCR; L/min) and renal reabsorption of urea.

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 cows were selected based on BW, parity, DIM, and milk production. At the start of the experiment, the BW of the cows was 655±45 kg, parity 2.8±0.97, DIM 190±41 d and milk production 28.1±3.23 kg/d (values expressed as means±SD). Eight cows were

housed in a free stall barn with cubicles on slatted floors and the other four cows were housed in a tie stall in order to quantitatively collect urine and feces. Groups of four cows were randomly assigned to a treatment according to a triple 4 × 4 Latin square. Treatments consisted of 4 dietary levels of sodium (3, 9, 14, and 19 g Na/kg DM) where levels of sodium were established through varying the dietary content of sodium chloride. The range in dietary Na levels was larger than observed in practice in order to augment variation in urine production, MUN, and renal characteristics. The large range in dietary Na levels is required to detect significant differences between treatments and to clearly show relationships between dietary characteristics, MUN, and urinary excretion of urea. The ingredients and chemical composition of the diets is presented in Table 1. During a two week adaptation period before the commencement of the study, cows were fed the ration with the lowest Na-content. The first nine days of this adaptation period, cows had ad libitum access to the low Na-content feed to determine feed intake. During the last five days of the adaptation period until the end of the experiment, cows were fed at 95% of ad libitum excluding salt addition. After this two week adaptation period, each period in the Latin square design lasted seven days where the first five days were used for adaptation and day six and seven for sample collection. Cows were milked twice daily at 05:00 h and 17:00 h throughout the experiment. During the non-collection days (d 1-5), cows were fed two equal meals, twice daily at 05:00 h and 17:00 h while during collection days (d 6-7), 75% of the daily feed allowance was provided in eight equal meals every two hours starting at 05:00 h until 19:00 h to minimize variation in PUN and MUN caused by variation in feed intake within day. At 21:00 h, the remaining 25% of the total daily feed allowance was provided. Daily individual feed intake in the tie stall was determined by subtracting the quantity of orts from the quantity of feed supplied. In the free stall barn, individual feed intake was registered with a computer controlled Hokofarm RIC system (Insentec, Marknesse, the Netherlands) and similar to collection days in the tie stall, allowance to feed was in equal sized meals every 2 h. During the last 2 days of the adaptation period until the end of the experiment the rectal body temperature was measured during the morning and afternoon milking. Cows were considered healthy when feed intake, milk production, and rectal body temperature were equal to the situation at the end of the adaptation period.

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

	Dietary Na-content (g/kg DM)			
Composition	**3**	**9**	**14**	**19**
Ingredients				
Corn silage[1]	720	709	698	687
Wheat straw, chopped	75	73	72	71
Soybean meal	171	169	166	163
Limestone	9.2	9.1	8.9	8.8
Soybean hulls	7.6	7.5	7.3	7.2
Urea	4.5	4.4	4.4	4.3
Soybean oil	4.1	4.0	4.0	3.9
Feed salt[2]	4.1	20.1	35.6	50.6
Mineral premix[3]	4.2	4.1	4.1	4.0
Nutrients				
DM (g/kg feed)	450	458	459	461
CP	155	153	151	151
Ash	71	87	95	113
Crude fat	29	30	29	28
Starch	235	224	216	212
NDF	358	344	343	338
ADF	218	210	209	206
ADL	23	22	23	22
Ca	6.7	6.7	6.4	6.4
K	12.9	13.0	12.3	12.6
Na	2.8	9.2	13.7	19.3
Feeding value parameters				
DVE[4]	91	90	88	87
OEB[5]	21	21	20	20
NE_L[6] (MJ/kg DM)	6.76	6.66	6.56	6.47
Rumen degradable CP	102	100	99	98

[1]Corn silage (g/kg DM unless specified otherwise): DM, 359 g/kg; CP, 81; starch, 423; NDF, 301; ADF, 162; ADL, 15; ash, 44 (determined with NIRS; Blgg, Oosterbeek, the Netherlands).

[2]Composition of feed salt: ≥ 99.8% NaCl.

[3]Contained per kilogram of mix: 152 g Ca; 220 g Mg; 6 g S; 5,780 mg Cu; 2,900 mg of Mn; 5,782 mg Zn; 290 mg I; 87 mg Co; 72 mg Se; 1.444,000 IU vit. A; 346,560 IU vit. D3; 6,839 IU vit. E.

[4]Intestinal digestible protein (Van Duinkerken et al. 2011b).

[5]Rumen degraded protein balance (Van Duinkerken et al. 2011b).

[6]Net energy for lactation calculated with VEM (feed unit lactation) system (Van Es 1975).

Sample collection

The diets were prepared daily using of a paddle mixer (Holaras V.D.C. 1200) and a representative sample (~700 g) of each TMR was collected daily and stored at -20 ºC. Samples of each diet for each treatment week were pooled and stored at -20 ºC pending analyses. During the collection period (d 6-7), milk yield was determined and milk samples (10 mL) were obtained after each milking from the total quantity of milk. Milk samples were stored at 4 ºC in tubes containing sodium azide and analyzed within 5 days after collection.

The four tethered cows were fitted with indwelling Foley urine catheters (Ch 26, 100-cc balloon; Bard Benelux N.V., Nieuwegein, the Netherlands) attached to a collection vessel one day before each collection period (d 5) and was removed after each collection period. The animals were also fitted with two way blood sampling catheters (BD Careflow dubbel lumen, Beckton Dickinson BV, Breda, the Netherlands) in the jugular vein one day before the first treatment diet was fed, and catheters were kept open by a 33 U heparin /mL saline solution throughout the experiment. During the collection days (d 6-7) of each treatment period, blood samples (10 ml) were collected in heparin tubes every two hours from 05:00 h till 21:00 h and at 01:00 h. Samples were centrifuged at 3,00 × *g* for 15 min at room temperature within 2 h and blood plasma separated and collected before being stored at -20 ºC. Urine and feces were collected quantitatively during collection days (d 6-7) starting at 05:00 h. Urine production was recorded every 2 hours from 05:00 h till 21:00 h and at 01:00 h, weighed, sampled (6 ml) and the urine collection vessel replaced by an empty vessel containing 40 g of 48% H_2SO_4 from 05:00 h till 21:00 h whereas at 01:00 h the urine collection vessel was replaced by an empty vessel containing 80 g of 48% H_2SO_4. After weighing, the urine was added to a collection vessel in order to collect the urine of the complete collection day. Each time a urine sample was taken, the pH of the collected urine was measured and additional H_2SO_4 was added in case the pH value was above 3.0. At the end of each collection day (at 05:00 h), the total quantity of feces and acidified urine of each cow were weighed, thoroughly mixed, sampled (~200 g feces and 250 mL urine) and stored at -20 ºC pending further analysis. Water intake was recorded every 2 hours from 05:00 h to 21:00 h for each tethered cow during the collection days using individual water flow meters.

Analytical procedures

Dry matter content of TMR samples was determined by freeze drying (FTS Systems Dura-Dry MP, New York, USA) where after the freeze dried samples were ground in a cross beater mill (Peppink, Deventer, the Netherlands) to pass a 1 mm screen and analyzed for DM, ash, N, crude fat, NDF, ADF and ADL as described by Abrahamse et al. (2008). Starch was analyzed using enzymatic hydrolysis (ISO 15914; ISO 2004). Calcium, Na and K were determined according to ISO 6869 (ISO, 2000) by Pre-Mervo (Utrecht, the Netherlands). Milk was analyzed for fat, protein, lactose and somatic cell count as described by Abrahamse et al. (2008). Milk urea content was determined using the pH difference technique (ISO

14637; ISO, 2004). Urea concentration in blood plasma and acidified urine 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. Urea was analyzed in each blood plasma sample taken. Feces and acidified urine were analyzed for N by the Kjeldahl method with $CuSO_4$ as the catalyst (ISO 5983; ISO, 1997). 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 07:00 h, 13:00 h, and 17:00 h in order to obtain an average estimate of plasma creatinine over a day. Results of Kokkonen et al. (2001) from dairy goats indicate that these 3 sampling time points will yield a proper average creatinine concentration per day. Creatinine was analyzed as well in acidified urine from a complete collection day per cow. Specific gravity of acidified urine was determined by accurately weighing 1.0 ml of acidified urine using a calibrated pipet from a vortexed sample. Specific gravity of individual acidified urine samples was corrected for the effect of the sulfuric acid on the specific gravity of the urine and used to correct measured urine weight to urine volume.

Calculations

Clearance rate calculations were based on Berne et al. (1998). The creatinine clearance rate (CCR, L/min), representing the glomerular filtration rate (GFR) or the volume of blood being filtered by the kidneys was calculated:

$$\frac{\text{Total daily urinary creatinine excretion (mmol/d)}}{\text{Blood plasma creatinine concentration (mmol/L)}} /1440 \text{ (min/d)}$$

Urea clearance rate (UCR, L/min) was calculated as:

$$\frac{\text{Total daily urinary urea excretion (mg/d)}}{\text{Blood plasma urea concentration (mg/L)}} /1440 \text{ (min/d)}$$

Renal urea reabsorption ratio (RRR) was calculated as: 1 – (UCR/CCR). N balance was calculated as N-intake minus N excreted in milk, feces, and urine.

Statistical analysis

Effects of dietary Na-content on DMI, milk yield, milk composition, and MUN for all 12 cows was analyzed using a mixed linear model (employing the MIXED procedure of SAS version 9.2), with period, type of housing, and dietary Na-content included as fixed effects. Because of the repeated measurements set-up of the experiment, with repeated measurements

per cow over periods and per measurement period, we included random effects for cows and for period within cow to allow for correlation between the two consecutive collection days per cow in each period, with a compound symmetry covariance structure.

Effects of dietary Na-content on N-balance characteristics, and on MUN, PUN, urine production, urinary creatinine excretion, blood plasma creatinine concentration, CCR, and RRR, for the four tethered cows was analyzed with the same model as described above except that the fixed effect of type of housing was removed from the model. For all mixed models, 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. An indication of the proportion of variation explained by dietary Na-intake was obtained on some parameters by calculating an R^2 for Na-intake (R_{Na}^2) in the mixed linear model according to Edwards et al. (2008). A linear plateau model, adapted from Robbins et al. (2006), was used to model the relationship between CCR and RRR using the PROC NLIN procedure, METHOD=NEWTON. Significance was declared at $P < 0.05$, and tendencies at $0.05 \leq P < 0.10$.

Results

Cows, diets, milk production, and type of housing

All cows remained healthy throughout the experiment. The results of the first measurement period were discarded due to an erroneous weighting of the concentrate mixture into the TMR diet during two days and analysis of data in this study was based on data from the remaining three measurement periods. There were no feed refusals and N-intake across rations did not differ significantly (Table 2). The content of protein and lactose in milk, and somatic cell count did not differ among treatments whereas milk production and content of fat and urea in milk were affected by dietary Na-content. An increase in dietary Na-content from 3 to 19 g/kg DM was accompanied by a tendency (P=0.05) to a decrease in milk production by 4.7%, a linear (P<0.01) decrease in milk fat content of 6.5%, and a tendency for an increase (P=0.10) in milk protein content of 1.4% (Table 2). There was an effect of type of housing (P=0.01) on MUN but not on other parameters such as DMI, milk production, and milk composition. Cows in the free stall barn had a 1.52 mg N/dL higher MUN than cows in the tie stall.

Variation in MUN

A linear effect was found of dietary Na-content on MUN concentration (Table 2). The following relationship between MUN and Na-intake presented in Eq. [1] was obtained for all animals and averaging intercepts over the two housing conditions as there was a significant effect of housing:

$$\text{MUN (mg N/dL)} = 13.5 \pm 0.35 - 0.0068 \pm 0.00104 \times \text{Na-intake (g/d)}$$

(Tie stall + free stall barn housed animals based on n= 72 observations (12 cows × 3 periods × 2 days), R_{Na}^2 = 0.60) [1]

Urine production (Eq. [2], only tie housed animals) explained slightly more variation of MUN compared to Na-intake (Eq. [3], only tie-housed animals).

MUN (mg N/dL) = 13.0±0.47 – 0.046±0.0104 × urine production (kg/d)
(Tie stall housed animals based on n= 24 observations (4 cows × 3 periods × 2 days), R_{Na}^2 = 0.68) [2]

MUN (mg N/dL) = 12.8±0.44 – 0.0068±0.00157 × Na-intake (g/d)
(Tie stall housed animals based on n= 24 observations (12 cows × 3 periods × 2 days), R_{Na}^2 = 0.64) [3]

Table 2. Least square means for Na-intake, DMI, N-intake, milk production, and milk composition of lactating dairy cows from both the free stall barn and the tie stall housed animals based on n=72 observations (12 cows × 3 periods × 2 days) that were fed diets that differed in Na-content

	Dietary Na-content (g/kg DM)					P-value[2]	
Parameter	**3**	**9**	**14**	**19**	**SE[1]**	**L**	**Q**
Na-intake (g/d)	69	198	292	417	9.2	<0.01	0.18
DMI (kg/d)	21.0	21.4	21.6	21.6	0.34	0.08	0.74
N-intake (g N/d)	519	527	519	526	9.5	0.78	0.51
Milk yield and composition							
Milk yield (kg/d)	25.7	25.2	25.2	24.5	0.78	0.05	0.59
MUN[3] (mg N/dL)	13.3	12.1	11.3	10.8	0.32	<0.01	0.36
Fat (%)	4.43	4.40	4.32	4.14	0.135	<0.01	0.11
Protein (%)	3.64	3.64	3.66	3.69	0.059	0.03	0.23
Lactose (%)	4.41	4.36	4.42	4.41	0.049	0.55	0.31
SCC[4] (× 1000/ml)	233	314	316	240	98.8	0.99	0.35

[1]Standard error of the least square means.
[2]P-values of linear (L) and quadratic (Q) effect of dietary Na-content.
[3]Milk urea nitrogen.
[4]Somatic cell count.

N-metabolism

The effect of dietary Na-content on N-metabolism based on observations on the four tie-housed animals is shown in Table 3. No effect of dietary Na-content on daily N-intake, N-excretion in milk, and UUN was found. Excretion of N in feces showed a small, but significant negative linear relationship with dietary Na-content whereas UN and non-urea urinary N (NUUN; g N/d) had a small but significant positive linear relationship with dietary

Na-content. The positive linear relationship between NUUN and dietary Na-content (Table 3) largely explained the significant effect of dietary Na-content on UN as UUN was not affected by dietary Na-content. A positive N-balance of on average 43 g/d (8% of dietary N-intake) was observed.

Table 3. Least square means of N-intake and excretion of N in milk, urine, and feces, and on urea and non-urea-N excretion in urine of lactating dairy cows from the tie stall housed animals based on n=24 observations (4 cows × 3 periods × 2 days) that were fed diets that differed in Na-content

	Dietary Na-content (g/kg DM)					P-value[2]	
Parameter (g N/d)	**3**	**9**	**14**	**19**	**SE[1]**	**L**	**Q**
N intake	526	532	518	526	11.4	0.78	0.72
N in milk[3]	148	144	141	143	6.1	0.07	0.07
N in feces[4]	148	143	144	138	6.7	0.04	0.84
Urinary excretion							
Total N	189	185	203	205	5.9	0.03	0.81
Urea N	135	130	138	140	5.1	0.21	0.67
Non-urea N	53	56	65	66	1.4	<0.01	0.61
N balance[5]	44	51	33	42	11.5	0.65	0.62

[1]Standard error of the least square means.
[2]P-values of linear (L) and quadratic (Q) effect of dietary Na-content.
[3]Calculated as the sum of daily milk protein divided by 6.38.
[4]Quantity of feces produced multiplied by the N-content of fresh feces.
[5]Calculated as N-intake minus excretion of N in milk, feces, and urine.

Urinary N-excretion and MUN

No significant relationship between MUN and UUN (P=0.69) and between MUN and UN (P=0.13) was found (Fig. 1). Ratios of UUN:MUN varied from 10.9 to 14.4 at dietary Na-contents of 3 and 19 g/kg DM, respectively. A negative relationship was observed between MUN and NUUN (P<0.01) (Fig. 1).

Renal functioning

A positive linear relationship was found between dietary Na-content or Na-intake and urine production (Fig. 2; Table 4) and between dietary Na-content and water intake (Table 4). Per extra gram of Na-intake an extra 0.146±0.0172 kg of water was consumed. An increase in Na-content resulted in lowered PUN and plasma creatinine concentrations whereas CCR, RRR, and the quantity of creatinine excreted in urine was unaffected (Table 4). The following linear plateau relationship (adopted from Robbins et al., 2006) between CCR and RRR could be established (Eq. [4], Fig. 3):

$$RRR = L + U \times (R - CCR) \quad [4]$$

where (R - CCR) is defined as zero when CCR is larger than R, L = 0.610±0.0107, U (min/L) = -0.423±0.0926, and R (L/min) = 1.56±0.063; tie stall housed animals, n=24; R^2 Adjusted = 0.71.

The prediction of RRR was substantially improved (Eq. [5]) when the linear factor Na-intake was added to the linear plateau model described in Eq. [4].

$$RRR = L + A \times \text{Na-intake} + U \times (R - CCR) \quad [5]$$

where (R – CCR) is defined as zero when CCR is larger than R, L = 0.647±0.0172, A (d/g Na) = -0.00014±0.000055, U (min/L) = -0.445±0.0807, and R (L/min) = 1.56±0.052; tie stall housed animals, n=24; R^2 Adjusted = 0.77.

Table 4. Least square means for water intake, production and specific gravity of urine, creatinine excreted in urine, content of creatinine and urea in blood plasma, urea content of milk, creatinine clearance rate, and renal urea reabsorption of lactating dairy cows from the tie stall housed animals based on n=24 observations (4 cows × 3 periods × 2 days) that were fed diets that differed in Na-content

	Dietary Na-content (g/kg DM)					P-value[2]	
Parameter	**3**	**9**	**14**	**19**	**SE[1]**	**L**	**Q**
Water intake (kg/d)	61.7	82.1	90.9	115.7	5.02	<0.01	0.57
Urine (kg/d)	18.2	30.6	46.6	67.7	2.13	<0.01	0.27
Urine specific gravity (g/mL)	1.050	1.039	1.036	1.034	0.0013	<0.01	<0.01
Urine creatinine excretion (mmol/d)	123	114	134	128	13.8	0.65	0.84
Plasma creatinine (µmol/L)	56.0	53.0	50.3	47.8	1.79	<0.01	0.82
Plasma urea nitrogen (mg N/dL)	15.2	12.9	13.7	11.8	0.54	<0.01	0.89
Milk urea nitrogen (mg N/dL)	12.5	11.2	10.8	9.9	0.43	<0.01	0.90
Creatinine clearance rate (L/min)	1.49	1.54	1.88	1.89	0.232	0.16	0.76
Renal urea reabsorption ratio	0.58	0.53	0.61	0.54	0.037	0.76	0.62

[1]Standard error of the least square means.

[2]P-values of linear (L) and quadratic (Q) effect of dietary Na-content.

Condition of steady state

Water intake tended (P=0.10) to linearly increase from 05:00 h until 01:00 h the next day. Within this period two periods were observed with a numerical higher water intake, one between 07:00 h and 09:00 h and the other between 13:00 h and 15:00 h whereas between 01:00 h and 05:00 h, water intake was close to zero (Fig. 4). Numerically, two peaks in urinary urea nitrogen excretion were observed between 09:00 and 11:00 h, and between 13:00 and 15:00 h (Fig. 4). Level of PUN did not vary substantially during the day (Fig. 4). From

17:00 h until 05:00 h the next day, there was a linear increase (P<0.01) in urine production which was accompanied by a linear decrease (P<0.01) in urinary urea concentration.

Discussion

Variation in MUN

In various studies, almost invariably a positive relationship is observed between MUN and UN (Ciszuk and Gebregziabher, 1994; Jonker et al., 1998; Nousiainen et al., 2004) and between MUN and UUN (Burgos et al., 2007; Powell et al., 2011). However, in this study MUN was not positively related to UN and UUN. An increase in Na-intake increased UN and decreased MUN significantly, indicating the opposite effect of that observed in other studies. This effect of dietary Na-content on the relationship between MUN and UUN or UN shows that Na-intake should be considered when MUN is used as an indicator of UN or UUN. The effect of dietary Na-content on MUN probably was indirect by its effect on urine production level, which directly affects MUN and UUN:MUN ratio. Such an effect implies that a change in water intake, or a change in dietary intake of any mineral that stimulates water intake and urine production, will affect MUN as well. Such a direct relationship between water intake and urea level in blood plasma and/or milk seems apparent from other studies. Burgos et al. (2001) found that dehydration (50% of ad libitum water intake) resulted in a 58% increase of MUN whereas Weeth and Lesperance (1965) observed a 14% decrease in PUN in hydrated (150% of ad lib. water intake) animals. Urine production may also differ substantially between corn silage based and grass silage based rations due to a low and high mineral content of corn silage and grass silage, respectively. This was shown by De Campeneere et al. (2006) who observed levels of urine production of 14.4 and 35.0 kg/cow/d for cows fed 100% corn silage and 100% grass silage based rations, respectively. According to Eq [2] in the present study, this 20.6 kg/d higher urine production on the grass silage based ration will lead to a 1.0 mg N/dL lower MUN. Correcting MUN for the effect of urine production in the study of De Campeneere et al. (2006) reduces the UN:MUN ratio of the grass silage based diet from 13.9 to 12.4, which is closer to the UN:MUN ratio of 11.1 observed for the corn silage based ration. In another study, De Campeneere et al. (2009) observed a decrease in MUN from 15.4 to 11.4 mg N/dL when 500 g/d of NaCl (197 g/d of Na) was added to a corn silage based ration which is equivalent to a 2.0 mg N/dL decrease in MUN per 100 g/d Na supply. This is a much stronger decrease than the decrease of MUN of 0.68 mg N/dL per 100 g Na/d established in the present study. The reason for this difference in the size of effect of Na on MUN is not clear, but it is not related to differences in N-intake, milk production, and manure N-excretion, as these were similar in both studies.

In practice the variation in dietary Na content is less than that in the present study. The NRC (2001) recommendation of Na for lactating dairy cattle is 2.2 g Na/kg DM but a maximum performance of dairy cows at dietary Na contents of 8.0 g Na/kg was reported. Assuming these differences in Na levels as the range in dietary Na contents observed in practice, the

difference in Na content between 2.2 and 8.0 g Na/kg at a DMI of 20 kg/d would result in a calculated MUN difference of around 0.79 mg N/dL or 7% of the average level of MUN observed in this study. However, differences in urine volume in practice may be far larger than observed within this limited range (e.g., De Campeneere et al., 2006) and minerals other than Na will affect urine volume.

Type of housing

Although the effect of dietary Na-intake on MUN was similar between cows in the tie stall (MUN = -0.0068 × Na-intake (g/d)) and cows in the free stall barn (MUN = -0.0067 × Na-intake (g/d)), a significant difference in MUN of 1.52 mg N/dl was observed between the two types of housing. The reason for this difference in MUN is unknown. There were no differences in DMI and N-excretion in milk between the two types of housing. Feed intake patterns were similar for cows in the free stall barn and in the tie stall. One might speculate that cows in the free stall barn had more physical exercise, affecting the overall body metabolism and urea concentration in plasma and milk as well.

Urine production and water intake

In the present study, a significant linear positive relationship was established between Na-intake and urine production suggesting that 0.136 kg of urine is produced per extra gram of Na-intake (Fig. 2). This value compares reasonably well to the estimate of Bannink et al. (1999) of 0.115 kg urine per g Na-intake based on a meta-analysis on trials having a wide range of dietary treatments. Likewise water intake was positively related to Na-intake (Table 4). The estimated slope (0.146 kg of water/g Na-intake) is within the range reported in literature (0.054 kg of water/g Na-intake; Murphy et al. (1983); 0.406 kg of water/g Na-intake; Meyer et al. (2004).

N-metabolism

The dietary Na-content did not affect UUN whereas NUUN was significantly affected (Table 3). We hypothesize that the highly significant and positive relationship between Na-intake and NUUN was the result of an increase in urinary excretion of purine derivatives, originating from absorbed rumen microbial nucleic acids. The increased water consumption with increased dietary Na levels observed in this study (Table 4) may have resulted in an increase of rumen liquid fractional passage rate, and consequently an increased efficiency of microbial growth rate and outflow of microbial protein to the intestine (Harrison et al., 1975; Dijkstra et al., 1998), which in turn led to an increase in intestinal absorption of derivatives of microbial nucleic acids, resulting in an increased NUUN. The negative relationship between MUN and NUUN (Fig. 1) is in accordance with the afore mentioned effect of Na-intake on NUUN. In contrast to this hypothesis, however, no effect of mineral intake on urinary allantoin excretion

in sheep was found by Houtert and Leng (1991), indicating that microbial protein synthesis was unaffected by level of salt intake.

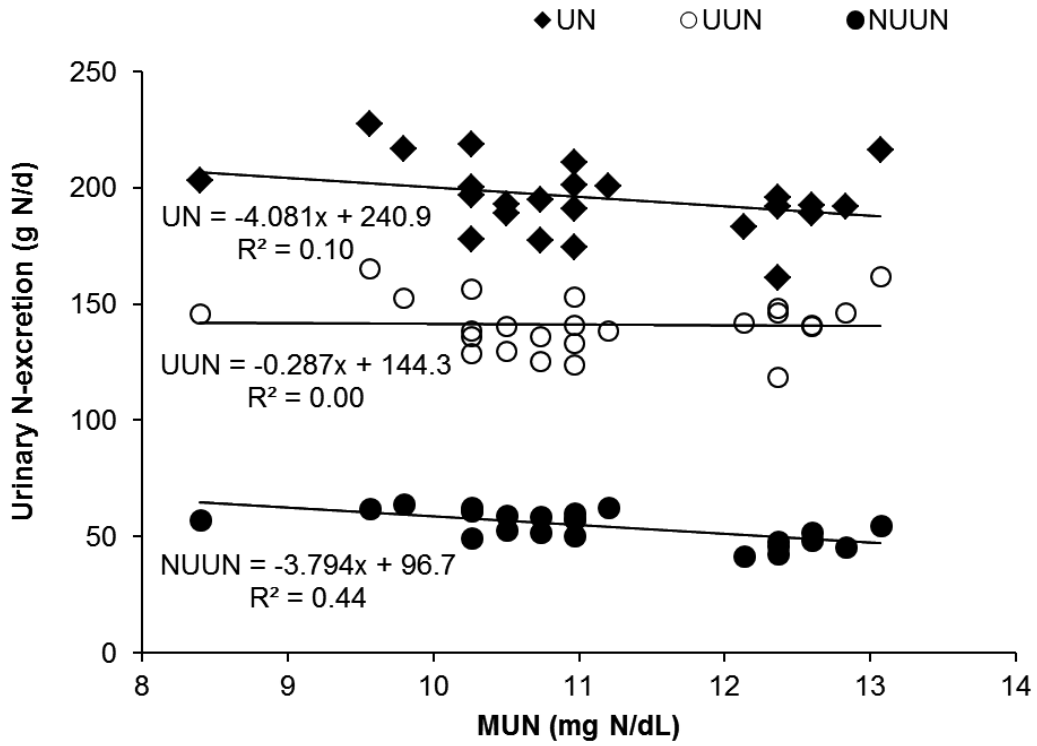

Figure 1. Relationship between milk urea nitrogen content (MUN; mg N/dL) affected by dietary Na consumption and excretion of urinary N (UN; g N/d), urinary urea-N (UUN; g N/d), and non-urea urinary N (NUUN; g N/d) for the tie stall housed animals based on n=24 observations (4 cows × 3 periods × 2 days). Each regression line is based on simple regression.

The positive N-balance of 43 g N/d on average (8% of N-intake) is in line with the average N-balance (39 g N/d) reported by Spanghero and Kowalski (1997) in a review on dairy cattle N-balance trials. Spanghero and Kowalski (1997) observed a positive relationship between N-balance and dietary N-availability. A similar relationship was observed in the present study where 46% of the variation of N-balance (ranging from 7 to 74 g N/d) could be explained by the effect of digestible N-intake. In this study, BW of the cows in the tie stall increased with 15±14.7 kg during the total period of the experimental period of six weeks, which is unlikely to account for the positive N-balance. Unaccounted N-losses that result in a positive N-balance can be ascribed to a combination of factors such as gaseous N-formation (Costa et al., 1968), excretion of N in urine as nitrate that is not detected by the Kjeldahl method (Young et al. 1981), and volatile losses of ammonia from feces and dermal and scurf losses (Spanghero and Kowalski, 1997). However, the possibility of an unaccounted N-loss through gaseous N was challenged by Rasch and Benevenga (2004) who could not account for the loss of 6.7%

of infused ^{15}N L-alanine via gaseous N in piglets or via other routes of excretion. Similarly, Spanghero and Kowalsky (1997) concluded that volatile N losses from feces and urine, errors in milk N-analysis, and the dermal and scurf N-losses, together could not fully explain the high N-balance observed.

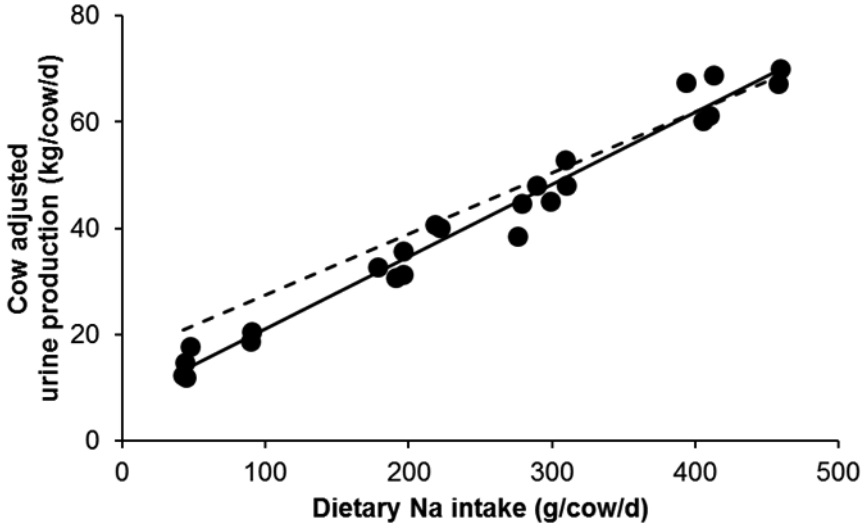

Figure 2. Effect of Na-intake on urine excretion for the tie stall housed animals based on n=24 observations (4 cows × 3 periods × 2 days). Urine excretion (kg/cow/d) = 7.5±4.33 + 0.136±0.0143 × Na-intake (g/cow/d) R_{Na}^2 = 0.92. Dashed line is urine excretion as predicted by the regression equation derived by Bannink et al. (1999).

Renal functioning

A positive linear relationship was observed between Na-intake and urine production (Fig. 2). The renal mechanism of increased urine excretion after an increase in water intake due to high levels of dietary salt, can be explained by 1) an increase in GFR caused by dilation of blood vessels, and 2) a decrease in renal reabsorption of water caused by a reduction in release of anti-diuretic hormone by the posterior pituitary. The negative correlation between Na-content and plasma creatinine concentration (Table 4) shows that at least the GFR (as indicated by CCR) is increased when salt intake increases. Apple et al. (1989) found that the coefficient of determination between GFR as determined by the inulin clearance rate (gold standard for determination of GFR) and CCR as measured by a modified rate Jaffé reaction was high (R^2=0.92) indicating that the reliability of CCR as an estimator for GFR is high. The increase in CCR may also have resulted in the decrease in PUN (due to the lower average retention time of urea in the blood) with increasing Na-intake, and subsequently may have decreased MUN. However, the potential effect of an increased urea flow to the kidneys on PUN was partially offset by an increase in RRR at the lower range of CCR (Fig. 3).

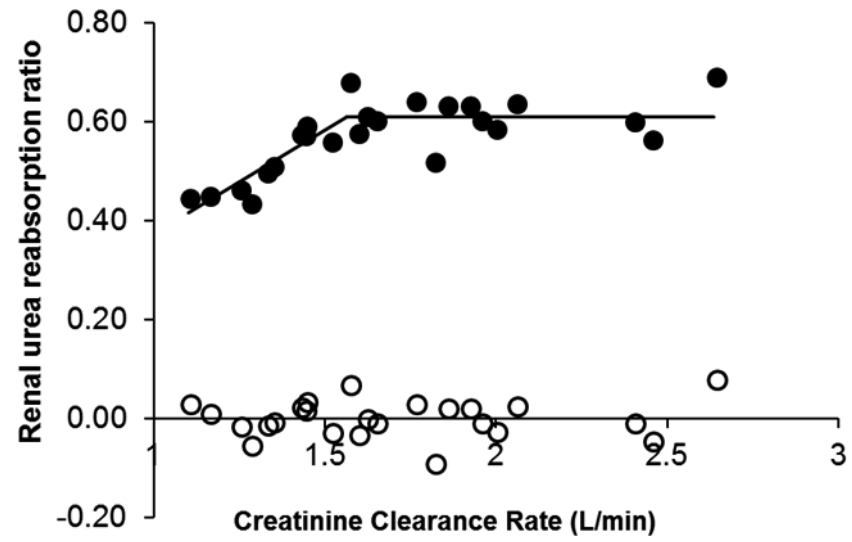

Figure 3. Effect of creatinine clearance rate (CCR; L/min) on renal urea reabsorption ratio (RRR) for the tie stall housed animals based on n=24 observations (4 cows × 3 periods × 2 days). L = 0.610±0.0107, U (min/L) = -0.423±0.0926, and R (L/min) = 1.56±0.063; R^2 = 0.72. The symbol ● represents the observed value whereas the symbol ○ represents the residual.

Three possible mechanisms may explain the effect of Na-intake on PUN. The first mechanism is that an increase in Na-intake will result in an increased GFR and an increase in urine production. This increase in GFR leads to a temporary increase in urea excretion in urine (if renal reabsorption of urea is not changed), resulting in a lower PUN. This lower PUN also lowers renal urea transport and UUN until the previous level of UUN is reached again. The second mechanism is that an increased renal urea excretion in the glomerular filtrate, due to an increase in GFR, is reduced by an increased renal reabsorption of urea from the glomerular filtrate. The third mechanism is related to the function of urea as an osmolite in the interstitial cells of the inner medulla of the kidney in concentrating urine (Schmidt-Nielsen et al., 1958; Schmidt-Nielsen and O'Dell, 1959; Bagnasco, 2000). As more urine is produced, the interstitial concentration of urea decreases resulting in less renal reabsorption of water and as a result an increase in urine production. This decrease in the interstitial concentration of urea is possibly caused by a reduction of RRR in the interstitial zone of the kidney and this reduction in RRR possibly affects PUN as well. However, the effect of interstitial urea concentration on PUN has not been tested. The results of this study indicate that renal regulation of urea excretion is a combination of at least the first and second mechanism described here.

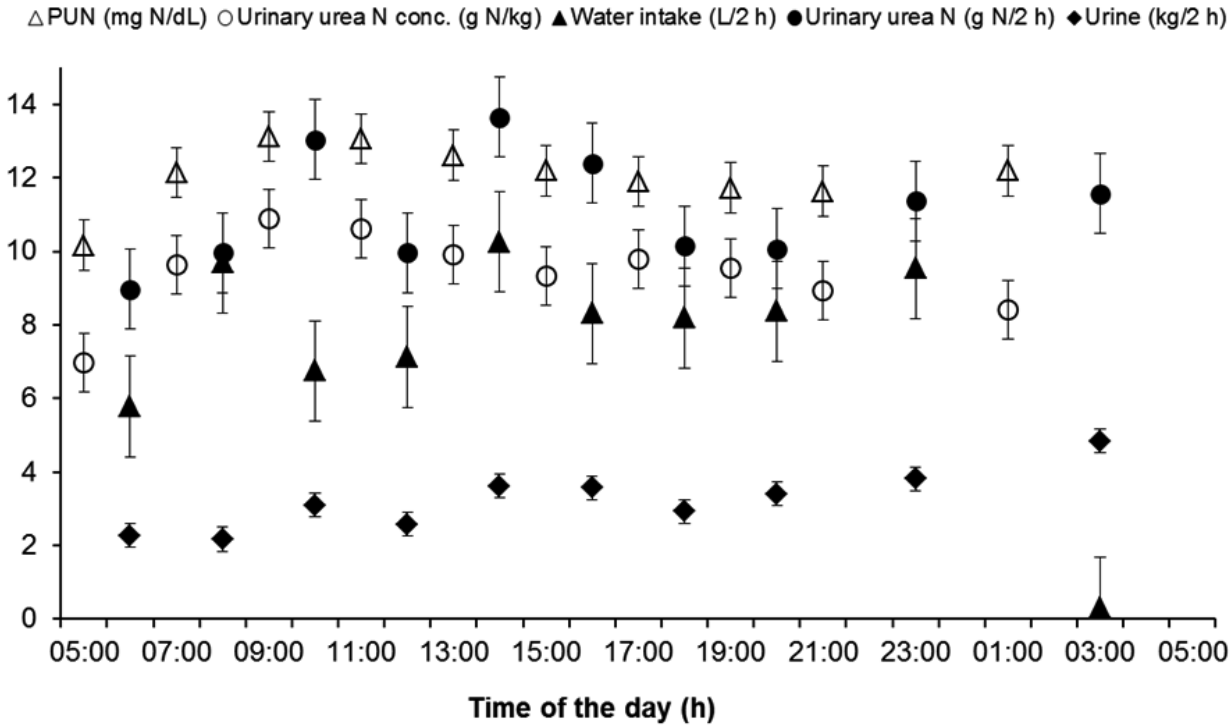

Figure 4. Effect of time of the day on urine urea N-excretion (g N/2 h), urine production (kg/2 h), water intake (L/2 h), and concentration of plasma urea-N (PUN; mg N/dL) and urine urea-N (g N/kg) for the tie stall housed animals based on n=240 observations (4 cows × 3 periods × 2 days × 10 observations per day), based on least square means that are corrected for ration and cow.

Conclusions

Dietary concentration of sodium chloride or the level of sodium chloride intake linearly increased urine production and total urinary N-excretion, and linearly decreased the concentration of milk urea nitrogen, but did not influence daily excretion of urinary urea nitrogen. Level of salt intake, should therefore be taken into account when milk urea nitrogen is used as an indicator of urinary urea nitrogen excretion by dairy cows. The kidneys adapt to an increase in glomerular filtration rate, caused by elevated levels of Na-intake, by increasing renal reabsorption of urea, thereby reducing the effect of salt intake on plasma urea nitrogen concentration.

Chapter 5

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

J.W. Spek[1,2], A. Bannink[2], G. Gort[3], W.H. Hendriks[1], and J. Dijkstra[1],

[1]Animal Nutrition Group, Wageningen University, PO Box 338, 6700 AH, Wageningen, The Netherlands
[2]Department of Animal Nutrition, Wageningen UR Livestock Research, PO Box 65, 8200 AB, Lelystad, The Netherlands
[2]Biometris, Wageningen University, PO Box 100, 6700 AC, Wageningen, The Netherlands

Journal of Dairy Science (2013) 96, 5734-5745

Abstract

Dietary protein and salt affect the concentration of milk urea nitrogen (MUN; mg N/dL) and the the relationship between MUN and excretion of urea nitrogen in urine (UUN; g N/d) of dairy cattle. The aim of the present study was to evaluate whether there is an interaction between dietary protein and sodium chloride (NaCl) content on MUN, UUN, on the relationship between UUN and MUN, on renal recycling of urea, and on urea transfer to the gastro intestinal tract. Twelve second parity cows with a BW of 645±37 kg, DIM of 146±29 d, and a milk production of 34.0±3.28 kg/d, of which eight 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 CP content as the main plot factor (116 and 154 g CP/kg DM) and dietary NaCl content as the sub plot factor (3.1 and 13.5 g Na/kg DM). Cows were fed at 95% of the average ad libitum feed intake of cows receiving the low protein diets. Compared to the low protein diets, the average MUN and UUN were, respectively, 3.90 mg N/dL and 45 g N/d higher for the high protein diets. Compared to the low NaCl diets, MUN was on average 1.74 mg N/dL lower for the high NaCl diets whereas UUN was unaffected. There was no interaction between dietary content of protein and NaCl on performance characteristics and 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 gastro intestinal 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. There was no interaction between dietary protein and NaCl content on urea entry rate and gastro intestinal urea entry rate or their ratio. The relationship between MUN and UUN was significantly (P=0.006) affected by the class variable dietary NaCl content: UUN = -17.7±7.24 + 10.09±1.016 × MUN + 2.26±0.729 × MUN (high NaCl); R^2 = 0.85. Removal of the MUN × 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. There is no interaction between dietary protein and NaCl content on performance, MUN, UUN, and renal urea recycling, nor on plasma urea entry rate and urea transfer to the gastro intestinal tract. For a proper interpretation of the relationship between MUN and UUN the effect of dietary NaCl should be taken into account, but there is no indication 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 laughing gas (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 N/d). Decreasing dietary crude protein content (CP; g/kg 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 environmental impact.

Milk urea-N (MUN; mg 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 body weight 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., 2012b). In a study by Spek et al. (2012b), 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. A number of 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 N/dL) and on renal handling of urea was studied by Thornton (1970) in two-year old steers. Addition of NaCl (200 g NaCl/d) reduced PUN with 42% from 3.48 to 2.02 mg N/dL at the high CP level (66.9 g CP/kg DM) whereas it reduced with only 19% from 1.15 to 0.93 mg N/dL at the low CP level (41.6 g CP/kg 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 suggests 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 three to four times higher than the 40 to 70 g CP/kg DM investigated by Thornton (1970). Furthermore, DMI in dairy cattle is substantially higher than DMI 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, on the relationship between MUN and UUN, and on the glomerular filtration rate and the reabsorption of urea from the glomerular filtrate. Another aim was to study the effect of dietary CP and NaCl on urea transfer to the gastro intestinal 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 eight with a rumen fistula, were selected based on milk production and presence of a rumen fistula. At the start of the experiment, the BW of the cows was 645±37 kg, DIM 146±29 d and milk production 34.0±3.28 kg/d (values expressed as means±SD). Cows were housed in a tie-stall in order to quantitatively collect urine and feces. Cows were blocked into three groups, according to milk production and presence of a rumen fistula. Cows within blocks were randomly assigned to one of four treatments. Treatments consisted of 2 dietary concentrations of protein (116 and 154 g CP/kg DM) and for each protein content two concentrations of NaCl (3.1 and 13.5 g Na/kg 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 sub plot factor. Each cow received in total two dietary treatments consisting of a low and a high dietary NaCl content, on either a low or high protein diet resulting in n = 24 observations. Because of practical limitations, measurements could be carried out for only six animals per day. For this reason the 12 cows were divided in two groups of 6 cows each. This resulted in a partial balanced design with four treatments and six cows per measurement period and a total number of four measurement periods. For each cow the total length of the study was 38 days. The first 13 days consisted of an adaptation period to the diet with ad libitum feed access and from day 14 onwards 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 two day measurement period (collection days 24 and 25) was followed by a 10 day adaptation period to the new diet (high or low NaCl) and followed by a second two day measurement period during (collection days 37 and 38).

Cows were milked twice daily at 05:00 h and 17:00 h throughout the experiment. During the non-collection days cows were fed two equal meals, twice daily at 05:00 h and 17:00 h, whereas during collection days 75% of the daily feed allowance was provided in eight equal meals every two hours, starting at 05:00 h until 19:00 h, to minimize variation in PUN and MUN caused by variation in feed intake within day. At 21:00 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.

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

	Low protein		High protein	
Composition	**Low NaCl**	**High NaCl**	**Low NaCl**	**High NaCl**
Ingredients				
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
Monocalciumphosphate	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
Nutrients				
DM (g/kg 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 parameters				
DVE[5]	69	67	105	102
OEB[6]	-9	-9	-9	-9
NE_L[7] (MJ/kg DM)	6.61	6.45	6.63	6.47
Rumen degradable CP	80	78	80	78

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

[2]Formaldehyde treated soybean meal (540 g CP/kg DM).

[3]Composition of feed salt: ≥99.8% NaCl.

[4]Contained per kilogram of mix: 48 g Ca; 0.8 g Mg; 6,263 mg Cu; 5,010 mg of Mn; 10,048 mg Zn; 501 mg I; 150 mg Co; 160 mg Se; 2.505,010 IU vit. A; 601,202 IU vit. D3; 6,263 IU vit. E.

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

[6]Rumen degraded protein balance (Van Duinkerken et al., 2011b).

[7]Net energy for lactation calculated with VEM (feed unit lactation) system (Van Es, 1975).

Sample collection

The diets were prepared daily by using a paddle mixer (Holaras V.D.C. 1200) and a representative sample (~700 g) of each TMR was collected daily and stored at -20 ºC. Samples of each diet for each treatment week were pooled and stored at -20 ºC pending analyses. During sample collection days, milk yield was determined and milk samples (10 mL) were obtained after each milking from the total quantity of milk. Milk samples were stored at -20 ºC in tubes containing sodium azide pending analyses.

One day before each sample collection period, cows used for sample collection were fitted with indwelling Foley urine catheters (Ch. 26, 30-cc balloon; Bard Limited, 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 two way blood sampling catheters (BD Careflow dubbel lumen, Beckton Dickinson BV, Breda, the Netherlands) in the jugular vein two days before cows were used for the first time of sample collection. Catheters were kept open by a 33 U heparin/mL saline solution throughout the experiment. During the sample collection days at 09:02 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 saline was injected intravenously for the low and high protein diets, respectively. With 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 07:00, 09:00, 09:22, 10:00, 11:00, 12:00, 13:00, 15:00, 17:00, and 21:00 h. These blood samples (10 ml) were collected in heparin tubes, centrifuged within 2 hours (3,000 × *g* for 15 min at 4 ºC), the blood plasma was separated and collected before being stored at -20 ºC. Urine and feces were collected quantitatively for 24 h during each collection day starting at 05:00 h. Production of urine and feces was recorded after each collection day, weighted, thoroughly mixed, sampled (~250 mL urine, ~200 g feces), and stored at -20 ºC. 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 in case 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 04:45 h and 16:45 h, rumen liquid samples (30 mL) were obtained from four cows with a rumen fistula, directly put in ice water and within 30 minutes centrifuged (3,000 × *g* for 15 min at 4 °C) and the supernatant stored at -20 ºC.

Analytical procedures

Dry matter content of TMR samples was determined after oven drying at 70 ºC during 24 hours 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; EC, 2009), ash (EC 152/2009; EC, 2009), N (ISO 5983; ISO,1997), crude fat (EC 152/2009; EC, 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 somatic cell count 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 08:00 h, 12:00 h, and 17:00 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). 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 08:00 h, 12:00 h, and 17:00 h, in order 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. To this end, the specific gravity of the urine produced is required, corrected for the effect of sulfuric acid added to the urine. Dividing the weight of urine by the sulfuric acid corrected specific gravity gives the volume of urine produced. The specific gravity of individual sulfuric acid corrected urine samples in this study was estimated based on the relationship between specific (sulfuric acid corrected) gravity of urine and the daily urine production in kg/cow/d determined in a previous study of Spek et al. (2012b; data on specific gravity unpublished; Fig. 1).

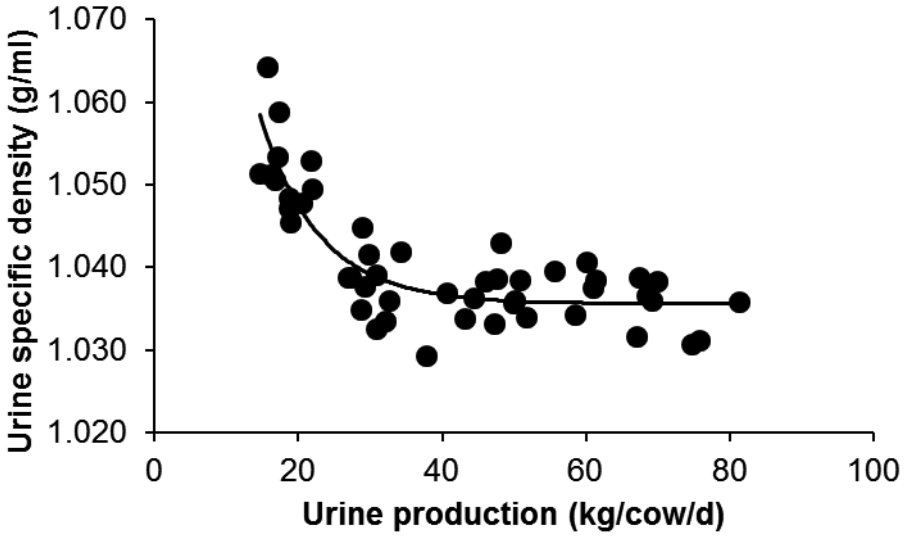

Figure 1. Relationship between urine specific density (g/ml) (after correction for added sulfuric acid during urine collection) and urine production (kg/cow/d), where variation in urine was caused by differences in NaCl intake (unpublished data of the specific gravity in urine in the study by Spek et al. (2012b)). Urine specific density (g/ml) = 1.0356±0.00091 + 0.134±0.0590 × exp(-0.121±0.0267 × urine production (kg/cow/d); $R^2 = 0.74$.

Ammonia (NH_3) in rumen liquid was determined colorimetrically using a spectrophotometer (Cary 50, Varian, Palo Alto, CA, USA) based on the Berthelot reaction as described by Searle (1984) after deproteinizing the supernatant by addition of 10% trichloroacetic acid. Enrichment of [^{13}C]urea in blood plasma was analyzed by 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 formula for calculation of the creatinine clearance rate (CCR, L/min), representing the GFR, and the formula for calculation of the urea clearance rate (UCR, L/min), and renal recycling ratio of urea excreted in the glomerular filtrate (RRR) are presented in a previous paper (Spek et al. 2012b). The N-balance was calculated as N-intake minus N excreted in milk, feces, and urine. Urea-N transfer to the gastro intestinal tract was calculated as the plasma urea-N entry rate (UER; g urea N/d) minus UUN and milk urea-N excretion rate (MUE; g urea-N/d):

$$\text{GER (g urea-N/d)} = \text{UER (g urea-N/d)} - \text{UUN (g urea-N/d)} - \text{MUE (g urea-N/d)} \quad [1]$$

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 urea-N) and then multiplied by 24:

$$\text{UER (g urea-N/d)} = K_{urea}\text{ (/h)} \times \text{PUP (g urea-N)} \times 24 \quad [2]$$

Correction of the [^{13}C]urea atom percentage for natural enrichment (measured immediately before infusion at 09:00 h) resulted in atom percent excess (APE; %) values for [^{13}C]urea. The K_{urea} was calculated by fitting an exponential model to the APE [^{13}C]urea enrichment data of plasma from 10:00 h until 21:00 h:

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

Where A is the estimated APE of [^{13}C]urea % at 09:02 h, the time of infusion of [^{13}C]urea.

The [^{13}C]urea observations at 09:22 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 (Fig. 2) resulting in a substantial 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 PUP was calculated by dividing the quantity of injected [^{13}C]urea by the APE [^{13}C]urea % fitted at 09:02 h using equation [3]:

PUP (g urea-N) = g [^{13}C]urea / (A/100)) × 28/60 [4]

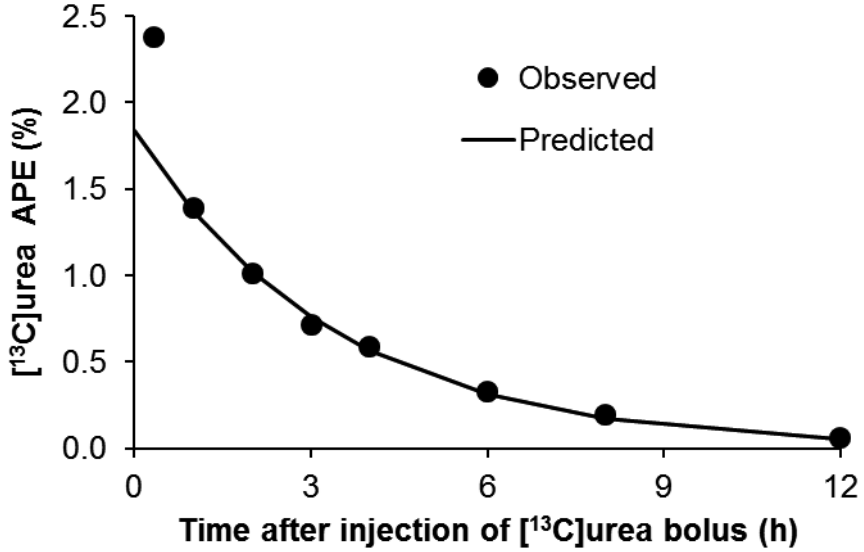

Figure 2. Example of an average result of a regression (excluding the data point at 0.37 h; see Materials and Methods for further explanation) of dilution of [^{13}C]urea expressed in atom percent excess (APE) percentage units in plasma after injection of a [^{13}C]urea bolus.

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 (package 9.2) with dietary protein, NaCl, and protein × NaCl interaction included as fixed effects, and cow nested within protein content as random effect. The dependent variables MUN, PUN, UN, 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.

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 (both UUN and MUN were not log transformed) was tested with the GLM procedure of SAS with UUN as the dependent variable and as independent variables MUN and the MUN × dietary NaCl interaction. Significance was declared at $P<0.05$, and tendencies at $0.05\leq P<0.10$.

Results

Cows, Diets, Milk Production, and MUN

During the measurement days there was one case of mastitis and one case of a blocked urine catheter, resulting in two missing values in the dataset and resulting in a dataset with n = 22 observations. The average difference in Na-intake between the high and low NaCl diets was 199 g Na/d (Table 2) and the average difference in N-intake between the high and low protein diets was 130 g N/d (Table 3). The DMI tended to be higher (P=0.065) for the cows receiving the high protein diets compared to cows receiving the low protein diets (Table 2). Compared to the low protein treatments, milk production at 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 somatic cell count 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 to the low protein treatments, MUN was 3.90 mg N/dL higher for the high protein treatment. Compared to the low NaCl treatments, MUN was 1.74 mg N/dL lower for the high NaCl treatments. There was no interaction (P=0.314) between dietary content of protein and NaCl on performance, milk composition, and MUN (Table 2).

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 cows[1]

Parameter	Low protein		High protein			P-value		
	Low NaCl	High NaCl	Low NaCl	High NaCl	SE	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[3] (mg 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[4] (× 1000/mL)	144	185	90	100	33.3	0.123	0.279	0.500
Rumen NH_3 (mg N/L)	32.9	36.7	45.4	36.2	4.25	0.257	0.554	0.182
ATTD[5] (%)	65.4	67.2	70.2	70.4	0.92	0.002	0.265	0.390

[1]Values are least square means.

[2]Fat and protein corrected milk; FPCM = (0.337+0.116 × fat % + 0.06 × protein%) × milk yield (kg/d).

[3]Milk urea nitrogen.

[4]Somatic cell count.

[5]Apparent total tract digestibility of DM.

UUN-MUN relationship

There was a significant interaction effect (P=0.006) of dietary NaCl (class variable) on the relationship between UUN and MUN: UUN (g N/d) = -17.7±7.24 + 10.09±1.016 × MUN (mg N/dL) + 2.26±0.729 × MUN (mg N/dL) × NaCl (high NaCl), R^2=0.85 (Fig. 3). Removal of the MUN × NaCl interaction term lowered the coefficient of determination from 0.85 to 0.77.

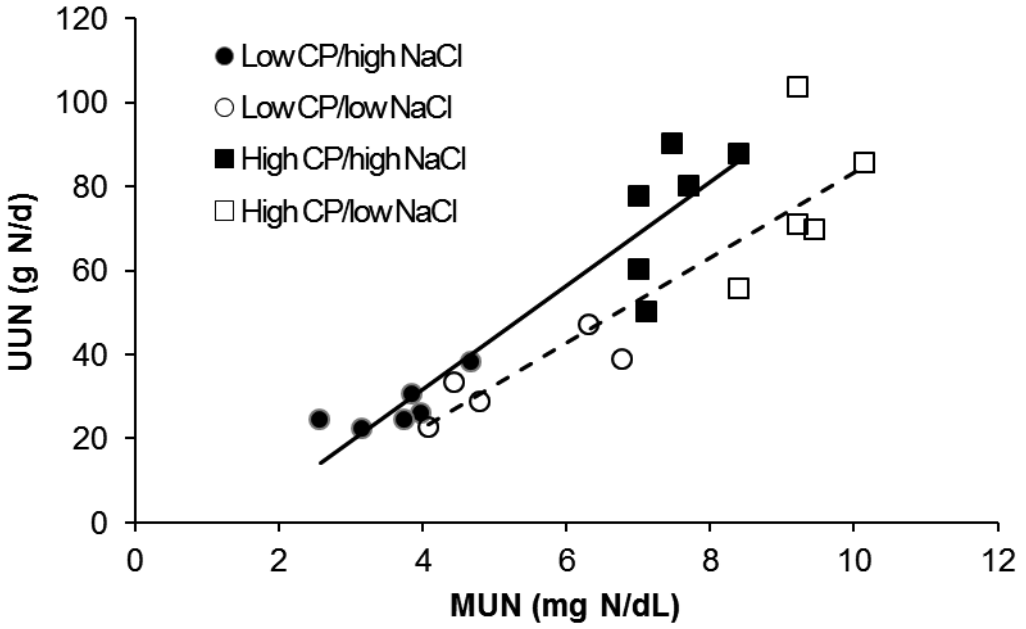

Figure 3. Relationship between milk urea nitrogen concentration (MUN; mg N/dL) and urinary urea nitrogen excretion (UUN; g N/d) for low NaCl (3.1 g Na/kg DM; dashed regression line) and high NaCl (12.9 g Na/kg DM; solid regression line) diets. UUN = -17.7±7.24 + 10.09±1.016 × MUN + 2.26±0.729 × MUN (high NaCl); R^2 = 0.85.

Nitrogen flows

Milk-N, fecal-N, and UN were all affected by protein content but not by NaCl content (Table 3). Compared to the low protein diets, high protein diets increased excretion of milk-N (19 g N/d), fecal-N (16 g N/d), UN (56 g N/d), UUN (46 g N/d), and non-urinary-urea-N (11 g N/d). Also, the average N-balance was 38 g 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 to 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 µmol/L), a 16% lower PUN (9.10 vs. 10.86 mg N/dL), and a 20% higher UUN:MUN ratio (8.91 vs. 7.40) (Table 4). Compared to the low protein diets, the high protein diets had a 68% higher PUN (12.51 vs. 7.45 mg N/dL), a 29% higher UUN:MUN

ratio (9.17 vs. 7.13), and a 45% increase in renal urea clearance rate (UCR; L/min) (0.421 vs. 0.290 L/min). There was 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).

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 cows [1]

	Low protein		High protein			P-value		
Parameter (g of N/d)	**Low NaCl**	**High NaCl**	**Low NaCl**	**High NaCl**	**SE**	**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

[1]Values are least square means.

[2]Calculated as N-intake minus excretion of N in milk, feces, and urine.

[3]Urinary urea-N excretion.

[4]Urinary non-urea-N excretion.

Urea transfer to the gastro intestinal tract

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 nor by dietary NaCl level. However, the GER:UER ratio, or the fraction of UER that is returned to the gastro intestinal tract (GIT), 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 N/d) and the concentration of milk urea nitrogen (MUN; mg N/dL), urea clearance rate (UCR), creatinine clearance rate (CCR), and renal recycling ratio of urea (RRR) in lactating dairy cows[1]

Parameter	Low protein		High protein		SE	P-value		
	Low NaCl	High NaCl	Low NaCl	High NaCl		Protein	NaCl	Protein × 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 (μmol/L)	68.4	65.3	64.3	62.1	2.34	0.252	0.080	0.745
PUN (mg 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

[1]Values are least square means.

[2]Excluding 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 cows[1]

Parameter	Low protein		High protein		SE	P-value[6]		
	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 urea-N)	30.6	25.5	48.9	45.6	2.48	<0.001	0.108	0.726
UER[4] (g urea-N/d)	226	199	315	292	11.8	<0.001	0.020	0.825
GER[5] (g 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

[1]Values are least square means.

[2]fractional disappearance rate of plasma urea-N.

[3]Plasma urea pool.

[4]Plasma urea entry rate

[5]Gastro intestinal 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 with 0.6 kg/d. A 6% higher ATTD was observed for the high protein diets. This finding indicates a reduced fermentation of feed in the gastro intestinal 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 a similar quantities of RDP and a similar rumen degradable protein balance (Table 1). The slightly negative dietary rumen degradable protein balance, indicating a slight shortage of NH_3 levels (32.9 – 45.4 mg N/L) that are below the levels of NH_3 considered to be sufficient for optimal microbial protein synthesis (Satter and Slyter, 1974). Milk production was higher for the high protein diets (25.6 kg milk/d) compared to the low protein diets (22.2 kg milk/d). This difference of 3.4 kg milk/d is slightly lower than the milk yield response to changes in dietary CP content predicted according to NRC (2001) (viz., 4.1 kg/d). However, differences in FPCM were much smaller and not significantly different. Compared to the low protein diets, the on average 15% higher N-excretion in milk for the high protein diets suggests that the low protein diets limited milk production. Reasons for this lower milk production are 1) a reduced ATTD of feed, resulting in less energy available for milk protein production and possibly a less efficient utilization of absorbed protein for milk protein synthesis; 2) a lower feed intake of 0.6 kg DM/d resulting in less energy and protein available for milk protein production; 3) a higher ratio of urea-N to total dietary N in the low protein diets (0.136) compared to the ratio of urea-N to total dietary N in the high protein diets (0.053) in this study as it is shown in a number of studies 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 N/dL per 100 g/d Na intake is similar to the decrease in observed PUN in the study of Weeth and Haverland (1961) of 0.85 mg N/dL per 100 g/d Na intake, but larger than the decrease in MUN of 0.68 mg N/dL observed in another recent trial by Spek et al. (2012b). No interaction between dietary NaCl and protein content on MUN was found. An interaction effect was anticipated based on a 42 and 19% decrease of PUN upon supply of 200 g 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 CP/kg DM for the high and low protein diets, respectively, were substantially lower than the 154 and 116 g CP/kg DM in the present study.

Possibly, 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 to the low salt diets, the higher (P=0.006) excretion of 2.26±0.729 g urinary urea-N per unit increase in MUN for the high salt diets is in line with results from Spek et al. (2012b) 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 NaCl intake resulting in urine levels of 13.1 to 33.1 kg/d are observed in practise 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, next to MUN, NaCl level into account is relevant for the dairy industry as 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 intake 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 due to the higher efficiency of microbial protein synthesis on true protein compared to 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, since 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 N/d, representing 8% of daily N-intake) and an even larger N-balance for the high protein diets (on average 66 g N/d, representing 14% of daily N-intake). This positive N-balance could not be ascribed to an 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 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 urine per g 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., 2012b) 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 ($P \leq 0.001$) and between dietary NaCl and PCC ($P=0.080$) can be explained by an increase in the glomerular filtration rate 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 to the low NaCl diets, no significant effects of dietary NaCl on CCR ($P=0.410$) were established. Previously, Spek et al. (2012b) also did not observe an effect of NaCl intake on CCR. In the study by Spek et al. (2012b), 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 GIT. However, in the present study a positive relationship between RRR and CCR was only found 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 for the low protein diets 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, 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 two UUN regulation mechanisms were also found in other studies (Schmidt-Nielsen and Osaki, 1958a; Schmidt-Nielsen et al., 1958b; Eriksson and Valtonen, 1982).

Urea transfer to the GIT

The fact that K_{urea} was negatively related to dietary protein content was unexpected as 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 urea-N) compared to the low protein diets (28.1 g urea-N) is 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 there was no significant effect of dietary NaCl on rumen concentration of NH_3. The fraction of UER returned to the GIT (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 to those predicted by Reynolds and Kristensen (2008) as shown in Fig. 4.

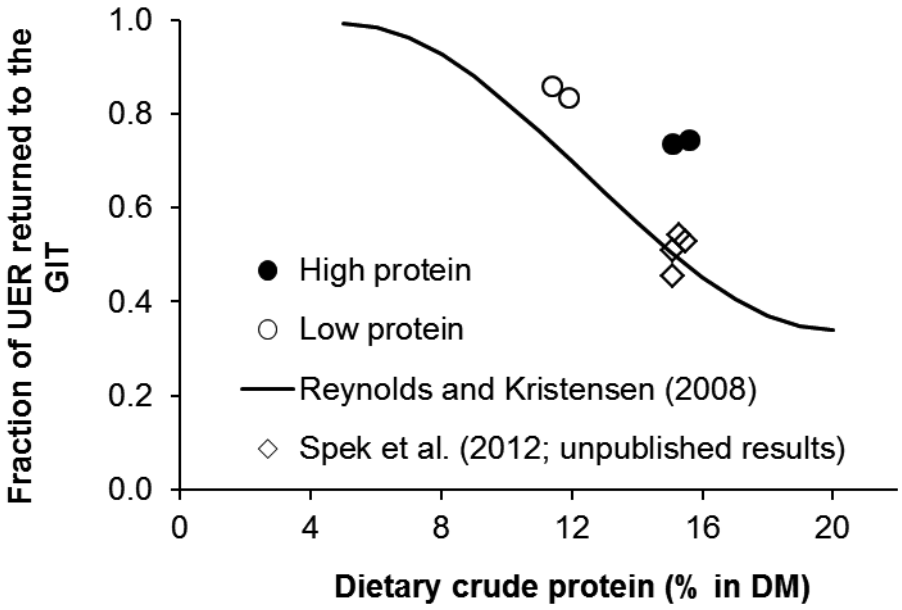

Figure 4. Relationship between dietary crude protein and the fraction of plasma urea entry rate (UER) that is returned to the gastro intestinal tract (GIT) in this study for high and low protein diets and in a previous study (Spek et al., 2012b). Solid line represents the relationship derived by Reynolds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

This difference is 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 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 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 as 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 beneath 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 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, due to 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, Fig. 4) nor in a former study by Spek et al. (2012b; unpublished results).

Conclusions

The MUN, PUN, and UUN were positively related to dietary protein content whereas the GER:UER ratio was negatively related to dietary protein content. The 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. It is concluded that for a proper interpretation of the relationship between MUN and UUN the effect of dietary salt should be considered.

Acknowledgements

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).

Chapter 6

Short Communication: Assessing urea transport from milk to blood in dairy cows

J.W. Spek[1,2], J. Dijkstra[1], J.J.G.C van den Borne[1], and A. Bannink[2]

[1]Animal Nutrition Group, Wageningen University, PO Box 338, 6700 AH, Wageningen, The Netherlands
[2]Department of Animal Nutrition, Wageningen UR Livestock Research, PO Box 65, 8200 AB, Lelystad, The Netherlands

Journal of Dairy Science (2012) 95, 6536-6541

Abstract

The concentration of urea in milk (MUC) has emerged as a potentially useful tool to predict urinary N-excretion. Various factors may affect the relationship between MUC and urinary N-excretion, including transport characteristics of urea from blood to milk and vice versa. The main objective of this study was to test whether there is substantial transport of urea from milk to blood in lactating dairy cattle. The sub-objectives were 1) to assess effects of various urea gradient levels between blood and milk on urea transport from milk to blood, and 2) to test the occurrence of urea transport between different compartments of the mammary gland such as the cistern and the alveoli. Urea transport was studied in two multiparous lactating HF cows (36.0±6.18 kg milk/d). In three separate trials, boluses of [^{15}N^{15}N]urea were injected in the cisterns via the teat canals at 20, 60, and 120 min before the 17:00 h milking at various levels of MUC and of blood plasma urea concentration (PUC).

In trial 1, a primed continuous infusion of urea (105 g + 20 g/h) into the jugular vein started at 05:00 h and stopped at 0, 1, 2, and 3 h before the 17:00 h milking on d 1, 2, 3, and 4, respectively. In trial 2, 5.5 g of urea was injected into the cisterns at 20, 60, and 120 min before the 17:00 h milking at d 5, 6, and 7, respectively. In trial 3, urea fluxes were measured without an experimentally induced gradient between MUC and PUC at d 8, 9, and 10, respectively. During milking, successive milk samples were taken from first to last milk. Blood and milk were analyzed for ^{15}N-urea enrichment. Levels of ^{15}N-urea in blood increased after injection of a [^{15}N^{15}N]urea bolus in milk, indicating urea transport from milk to blood. Between 21.0 and 35.3% of injected [^{15}N^{15}N]urea in milk was recovered after two hours. The fractional [^{15}N^{15}N]urea decline rate in milk varied between 0.0076 and 0.0096 /min. The level of MUC, rather than the concentration gradient between MUC and PUC, appeared to affect this fractional rate of decline. Enrichment levels of ^{15}N-urea in milk samples within a single milking showed that urea was transported from cistern milk to alveoli milk. In conclusion, the results indicate that transport of urea from milk to blood in lactating dairy cattle occurs and that urea is transported from cistern milk to alveoli milk.

Key words: milk urea, urea transport, mammary gland, dairy cattle

Short Communication

A positive relationship exists between the concentration of urea in milk (MUC; mg/dL) or in blood plasma (PUC; mg/dL) and excretion of nitrogen (N) in urine (Ciszuk and Gebregziabher, 1994). Because of this positive relationship, MUC has been used to predict urinary N-excretion and ammonia emission (Nousiainen et al., 2004; Burgos et al., 2007; Van Duinkerken et al., 2011a). However, the relationship is affected, among others, by diurnal dynamics in MUC, which in turn is largely influenced by feed intake pattern (Gustafsson and Palmquist, 1993) and transport characteristics of urea from blood to milk and vice versa.

Although it is well known that urea is transported from blood to milk, the reverse transfer of milk urea to blood is hardly investigated. Quantitative knowledge on urea diffusion from milk to blood may help in understanding the dynamics of MUC in dairy cows and its effect on the relationship between MUC and N-excretion in urine. Linzell and Peaker (1971) reported that in two goats, 82 and 70% of injected ^{14}C-urea in milk via the teat canals was recovered in the milk at one hour after injection. For high-yielding dairy cows with a much higher milk yield and a mammary gland structure that differs (e.g. the ratio of cistern milk to alveoli milk) from that in goats, urea disappearance from milk in the mammary gland has not been assessed. In their experiment, Linzell and Peaker (1971) also estimated the diffusion of lactose between different compartments in the mammary gland by injecting labeled lactose in the teat pouch and by collection of milk portions from cistern milk to alveoli milk. The quantity of injected labeled lactose recovered in residual milk after oxytocin injection was 7% and 14%, 10 and 20 min after injection, respectively. These observations suggest the occurrence of internal diffusion of lactose in the mammary gland, and, similarly, intra-mammary urea diffusion may occur as well. This route of diffusion of urea is of interest because it may impact urea transport between milk and blood, and consequently the relationship between MUC and urinary N-excretion.

The main objective of the current study was to test whether there is substantial transport of urea from milk to blood in lactating dairy cattle. The sub-objectives were 1) to assess effects of various urea gradient levels between blood and milk on urea transport from milk to blood in lactating dairy cattle, and 2) to test the occurrence of urea transport between different compartments of the mammary gland such as the cistern and the alveoli.

The study was approved by the Institutional Animal Care and Use Committee of the Animal Sciences Group, Wageningen University and Research Centre, Lelystad, the Netherlands. Three trials were conducted with two tethered, multiparous lactating HF cows (36.0±6.18 kg milk/d, 595±37.3 kg BW, and 66±8.5 DIM) fed a total mixed ration based on maize silage with 155 g crude protein per kg DM. To prevent variation in DM intake (and consequently variation in PUC and MUC) between days, cows were fed at 90% of their ad libitum intake which was determined during a period of two weeks before the start of the measurement period. On measurement days, feed was offered in equal portions every 2 h during daytime (05:00 – 17:00 h) to minimize fluctuations in PUC and MUC whereas on other days cows were fed equal portions at 05:00 h and 17:00 h. Cows were milked twice daily at 05:00 h

and 17:00 h. Three days prior to the first measurement day a double lumen semi-permanent catheter was inserted in the left jugular vein of the cows for collection of blood samples and infusion of urea.

In trial 1, it was tried to establish a positive gradient between MUC and PUC. A primed continuous infusion of urea (105 g at the start, continued with 20 g/h) into the jugular vein started at 05:00 h and stopped at 0, 1, 2, and 3 h before the 17:00 h milking on d 1, 2, 3, and 4, respectively. Two hours before milking, 50 mg of [^{15}N^{15}N]urea (98% ^{15}N enriched, Sigma-Aldrich, Zwijndrecht, the Netherlands) dissolved in 40 mL saline was injected in the cisterns of the mammary gland via the teat canals, i.e., 10 mL per cistern.

In trial 2, a positive gradient between MUC and PUC was established by direct injection of 5.5 g of urea dissolved in 40 mL saline into the cisterns via the teat canals (i.e., 10 mL per cistern) at 20, 60, and 120 min before the 17:00 h milking at d 5, 6, and 7, respectively. In addition, 70 mg of [^{15}N^{15}N]urea was simultaneously injected in the mammary gland cistern (i.e. 17.5 mg per cistern).

In trial 3, urea fluxes were measured without an experimentally induced gradient between MUC and PUC. Twenty mg of [^{15}N^{15}N]urea dissolved in 40 mL saline was injected into the cisterns (i.e., 10 mL per cistern) of the mammary gland at 20, 60, and 120 min before the 17:00 h milking at d 8, 9, and 10, respectively.

Related to sub-objective 2, milk was collected in trials 2 and 3 in portions of approximately 2 L using a WB HI / Pullout Tru-tester device (Tru-Test Ltd., Auckland, New Zealand) at the 17:00 h milking and each portion was sampled. Directly after milking, 20 IU of oxytocin was injected intravenously after which the residual milk was collected and sampled as well. In addition, a representative sample of the total collected milk was composed. Blood samples were taken in hourly intervals, starting at 3 h before milking until milking. Samples of milk and blood plasma were stored at -20 °C pending analysis.

Milk and blood samples were analyzed for urea concentration and for ^{15}N enrichment in urea. Milk samples were defatted by removing the fat after centrifugation at 9,000 × *g* for 15 min at room temperature and the defatted solution was deproteinized by centrifugation at 10,600 × *g* for 15 min at 4°C after precipitation of milk protein with trichloroacetic acid (10%). The resulting fat- and protein-free solution was used for analysis of urea concentration and ^{15}N enrichment in urea. Blood plasma samples were deproteinized by centrifugation at 10,600 × *g* for 15 min at 4°C after precipitation of plasma protein with trichloroacetic acid (10%). Urea concentration in blood plasma and milk was analyzed with the urea liquicolor test no. 10505 (HUMAN, Wiesbaden, Germany) which is based on measuring absorbance (578 nm) after a modified Berthelot reaction. Urea in the fat- and protein-free solution samples of milk was isolated by cation exchange chromatography (Dowex 50WX8-400 cation exchange resin, protonated with 0.1 M HCl solution) and elution with water. The first 4 mL of the eluent, containing lactose, was discarded, whereas the following 30 mL of the eluent, containing the majority of milk urea (>95%) was collected. This 30 mL of eluent was dried

with a rotary evaporator at 60 °C, re-dissolved in 1 mL of water, transferred into capsules of tin, and analyzed for total ^{15}N enrichment by EA-IRMS (EA model DP 200 series 2, Finnigan, Milan Italy; IRMS model DELTA S, Thermo Finnigan, Milan Italy). It was assumed that the ^{15}N enrichment in milk urea was entirely caused by the quantity of [$^{15}N^{15}N$]urea infused whereas the quantity of infused [$^{15}N^{15}N$]urea that is transported to the blood and returned to the milk was assumed to be negligible due to the strong dilution of ^{15}N urea in the total plasma pool.

Data were analyzed with the PROC GLM procedure from SAS in which cow and trial were added as class variables whenever data from cows or trials were pooled whereas all other factors such as PUC, MUC, ^{15}N-urea enrichment percentage in milk, and time were added as fixed continuous variables. In trial 2 and 3, the recovered ^{15}N urea in milk was used to estimate the fractional disappearance rate of injected [$^{15}N^{15}N$]urea according to an exponential model with the NLIN procedure of SAS.

In trial 1, between 24.9 and 35.3% of [$^{15}N^{15}N$]urea injected in the cisterns of the mammary gland at 2 h before milking was recovered in the milk (Table 1).

Table 1. Results from Trial 1. Effect of the time interval between ending an intravenous urea infusion and milking on urea concentrations in the milk and the blood, on the urea concentration gradient between plasma and milk urea, and on the 2 h recovery of an intra-mammary bolus injection of [$^{15}N^{15}N$]urea in milk (n=2, data presented as means±SD).

Time between the end of urea infusion and milking (h)	MUC[1] (mg/dL)	PUC[2] (mg/dL)	Urea concentration gradient (mg/dL)	^{15}N-urea recovered in milk[3] (%)
0	69.7 ±3.22	77.1 ±3.56	7.4 ±6.78	35.3 ±1.96
1	58.1 ±0.59	60.2 ±6.59	2.1 ±7.18	27.3 ±1.23
2	59.1 ±2.24	56.5 ±2.78	-2.6 ±0.53	28.9 ±1.16
3	53.6 ±1.84	51.4 ±2.79	-2.1 ±0.59	24.9 ±7.63

[1]MUC: milk urea concentration.

[2]PUC: blood plasma urea concentration. Values of PUC are averages of two PUC values, one value of PUC at 1 h before milking and a second value of PUC at the moment of milking.

[3]The percentage of an intra-mammary bolus of [$^{15}N^{15}N$]urea at 2 h prior to milking that is recovered in milk.

This indicates urea transfer from the mammary gland into other body fluids such as blood plasma. The recovery of ^{15}N-urea in milk was highest (35.3%) for the treatment in which intravenous urea infusion continued until milking, and lowest (24.9%) when infusion of urea was stopped at 3 h before milking, but these differences were not significant (P=0.22), possibly due to the small number of animals in this experiment. The results of trial 1 indicate a positive relationship between recovery of ^{15}N-urea in milk and the level of MUC (R^2=0.73, P=0.02). Recovery of ^{15}N-urea in milk was not related to the urea concentration gradient

between plasma and milk urea (R^2=0.10, P=0.82). Standard deviations of PUC and MUC in trial 1 (Table 1) varied between 0.59 and 3.22 mg/dL for MUC and between 2.78 and 6.59 mg/dL for PUC. Reasons for this range in standard deviations might, among others, result from differences in water intake between cows, as there are some indications that water intake affects MUC (Burgos et al. 2001; J.W. Spek, unpublished data). However, water intake was not measured in this study.

Trials 2 and 3 confirmed the transfer of urea from the mammary gland into plasma observed in trial 1. Between 21.0 and 34.3% of the labeled urea injected into the mammary gland was recovered in milk at 2 h after injection in trials 2 and 3 (Fig. 1). The average MUC was substantially higher in trial 2 (43.3±5.49) than in trial 3 (20.1±2.33), which was a consequence of injecting a bulk of unlabeled urea next to injection of the labeled urea in the cisterns in trial 2. The average PUC was similar in trial 2 (23.3±2.37) and trial 3 (22.8±3.93), resulting in a larger gradient between PUC and MUC in trial 2.

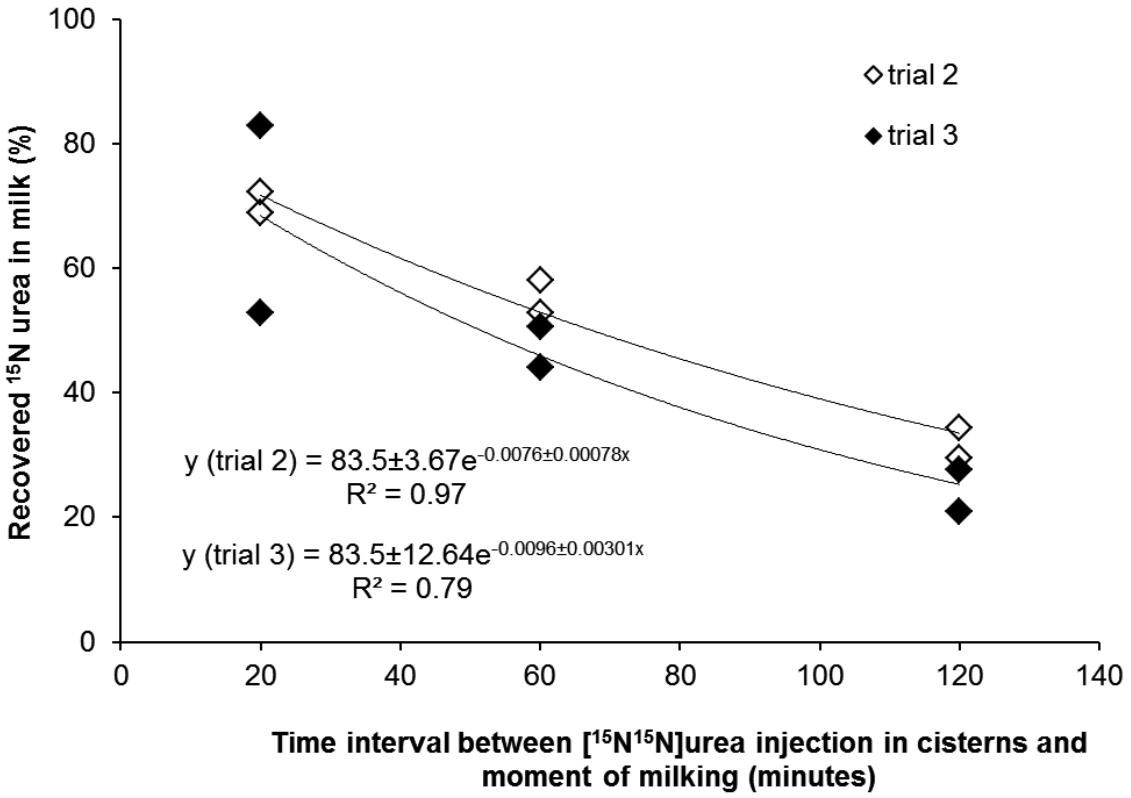

Figure 1. Effect of time interval (20, 60, or 120 min) between [^{15}N^{15}N]urea injection into the cisterns and milking on recovered ^{15}N urea in milk (% of injected) in trial 2 and 3. In trial 2, 70 mg [^{15}N^{15}N]urea and 5.5 gram unlabeled urea was injected in the cistern of the mammary gland via the teat canals at 20, 60, and 120 min before milking at 17:00 h whereas in trial 3, 20 mg [^{15}N^{15}N]urea (no unlabeled urea) was injected in the cistern of the mammary gland at 20, 60, and 120 min before milking at 17:00 h.

In line with results from trial 1, results from trials 2 and 3 indicate that more ^{15}N urea was recovered in milk with a higher MUC and independent of the gradient between MUC and PUC. With lower MUC (trial 3 vs. trial 2), the percentage of ^{15}N urea recovered in milk after 60 and 120 min was reduced (-18%, P=0.02) whereas the concentration gradient between PUC and MUC had no effect on fractional urea transport from milk to blood (R^2=0.07, P=0.56). These results indicate a negative association between MUC and fractional urea transport from milk to blood. The rise in ^{15}N enrichment of plasma urea after [$^{15}N^{15}N$]urea injection in the mammary gland in trial 3 (Fig. 2) indicates that transport of urea from mammary gland to blood occurred.

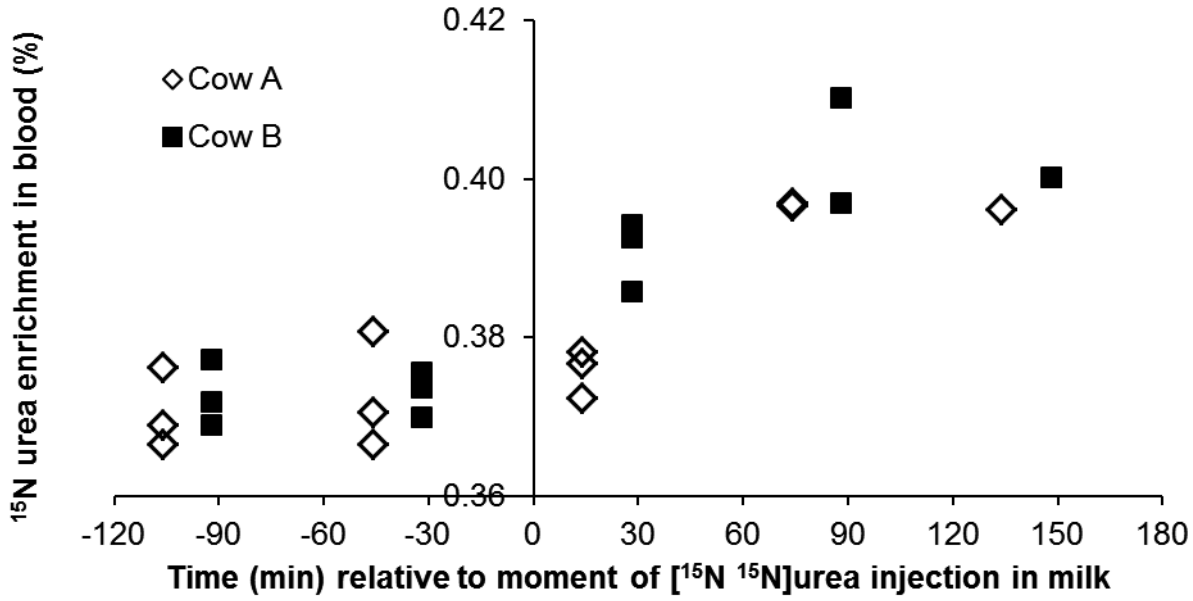

Figure 2. ^{15}N urea enrichment in blood before and after injection of [$^{15}N^{15}N$]urea in milk in trial 3

For day 9, 55% of the injected [$^{15}N^{15}N$]urea was recovered in the milk. Based on 1) the increase in ^{15}N enrichment of urea in blood plasma, 2) level of PUC, and 3) an assumed urea distribution volume of 68% of BW as found by Agnew et al. (2005), around 29% of the injected [$^{15}N^{15}N$]urea in the mammary gland was recovered in blood plasma. The exponential disappearance curves of ^{15}N urea in milk (Fig. 1) show intercept values of 83.5%, indicating an unaccountable loss of injected [$^{15}N^{15}N$]urea in the order of magnitude of 16% as well. The loss of injected labeled urea may be due to an immediate distribution within the mammary gland urea space volume which is larger than the milk volume, or some conversion of absorbed urea into ammonia upon transport into the gastro intestinal tract. Given the very low amounts of ammonia in milk, significant conversion of urea by urease activity in milk is unlikely.

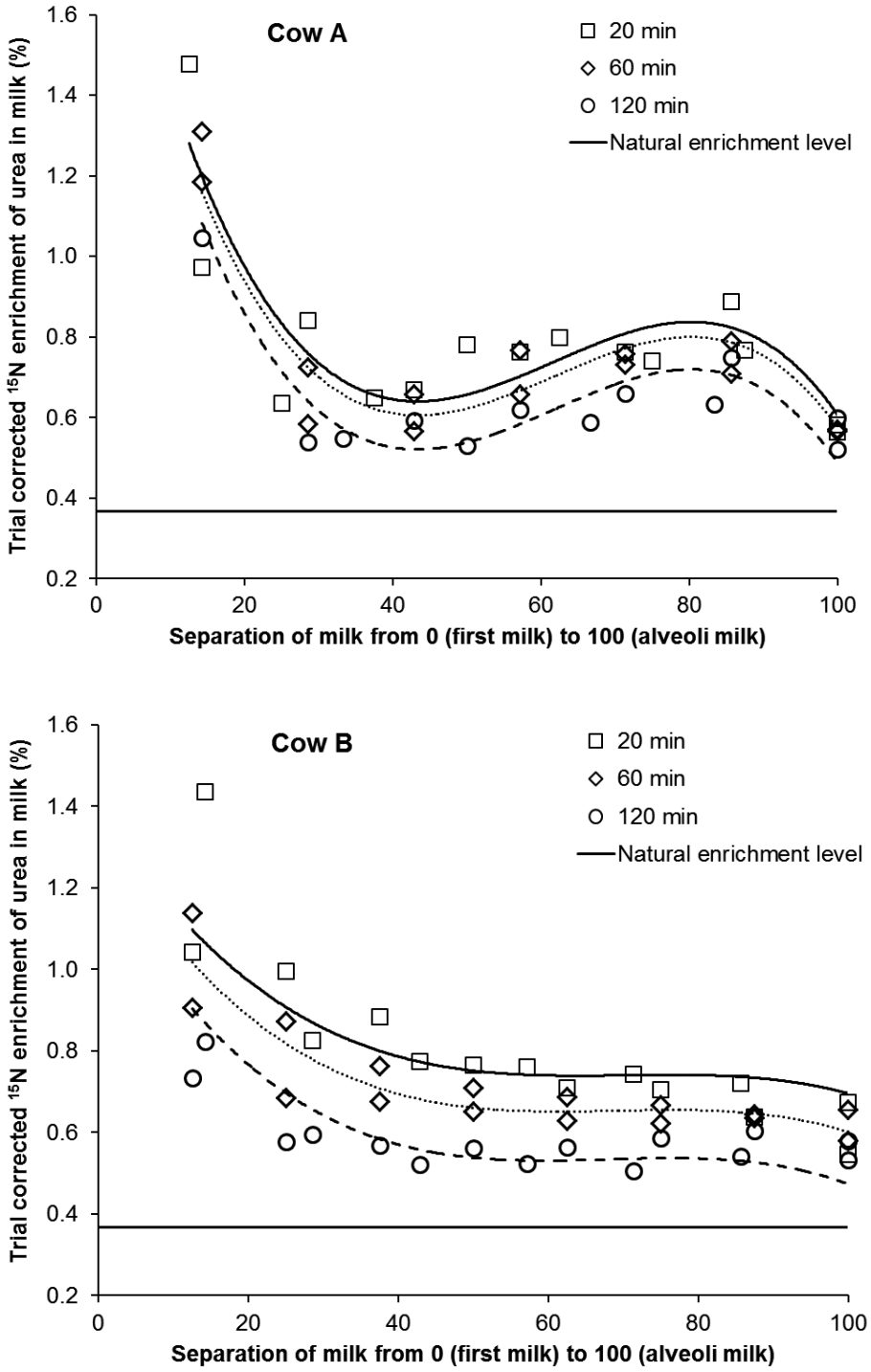

Figure 3. Trial corrected enrichment of ^{15}N urea in milk from the first (cistern) to the last milk (alveoli) based on data from trial 2 and 3 and presented individually for cow A and B. [$^{15}N^{15}N$]urea was injected in the cisterns at 20, 60, and 120 minutes before milking at three consecutive days. Third order polynomial models were fitted to the data (estimates presented in Table 2) resulting in R^2 values of 0.84 and 0.85 for cow A and B, respectively. Solid line = 20 min, dotted line = 60 min, and the dashed line = 120 min.

In Figure 3, the ^{15}N urea enrichment in the milk fractions from first (cistern) to last (alveoli) milk are plotted for each of the three moments before milking (20, 60, and 120 min) when a bolus of [$^{15}N^{15}N$]urea was injected in the cisterns of the mammary gland.

Table 2 presents the parameter estimates of 3rd order polynomial models fitted with the PROC GLM procedure of SAS for cow A and B in Fig. 2. The results presented in Fig. 2 indicate that injected [$^{15}N^{15}N$]urea in the cisterns of the cow diffuses towards the alveoli. Especially for cow A, the ^{15}N urea enrichment curve suggests that urea transport in milk cannot simply be explained by a single compartment model as indicated by a best fit obtained with a 3rd order polynomial model represented in Table 2.

Table 2. Model parameters of regression of a third-order polynomial model to ^{15}N enrichment distribution in milk from first to last milk in cow A and B separately, based on observations from trial 2 and 3 (regression results shown in Figure 2).

	Cow A			**Cow B**		
Parameter	**Estimate**	**SE**	**P**	**Estimate**	**SE**	**P**
Intercept	1.89	0.116	<.001	1.07	0.098	<.001
Trial 2	0.13	0.029	<.001	0.254	0.0267	<.001
Trial 3	0					
20 min	0.119	0.0358	0.002	0.226	0.0331	<.001
60 min	0.0876	0.03634	0.022	0.1294	0.03252	<.001
120 min	0			0		
X*	-0.0808	0.00777	<.001	-0.0301	0.00686	<.001
X^2	0.00143	0.000152	<.001	0.00044	0.000137	0.003
X^3	-7.73E-6	0.88E-6	<.001	-2.13E-6	0.80E-6	0.011

*The parameter X can vary from 0 (first milk) to 100 (last milk) and it indicates a sub-compartment of milk in the mammary gland, ranging from the cistern (first milk) to the alveoli (last milk).

Compared to cow B, an increase of ^{15}N urea enrichment in the second half of the milk obtained during a milking in cow A was observed (Fig. 3). These differences between cow A and B may indicate physiological differences in mammary gland morphology such as blood circulation, degree of urea diffusion between blood and milk of the various mammary gland compartments, and/or milk release characteristics from the different quarters of the udder.

In conclusion, our results indicate that transport of urea from milk to blood in lactating dairy cattle occurs and that this transport is related to MUC level. Moreover, results indicate that urea is transported intra-mammary from cistern to alveoli milk.

Chapter 7

Influence of milk urea level on urea transfer from milk to blood in dairy cows

J.W. Spek[1,2], J. Dijkstra[1], and A. Bannink[2]

[1]Animal Nutrition Group, Wageningen University, PO Box 338, 6700 AH, Wageningen, The Netherlands
[2]Department of Animal Nutrition, Wageningen UR Livestock Research, PO Box 65, 8200 AB, Lelystad, The Netherlands

To be submitted

Abstract

A positive relationship exists between the concentration of urea nitrogen in milk (MUN; mg N/dL) and in blood plasma (PUN; mg N/dL) and excretion of nitrogen (N) in urine. Because of this positive relationship, MUN has been proposed as an indicator of urinary N-excretion and of ammonia emission related to this. The relationship between MUN and urinary N-excretion is affected, among others, by diurnal dynamics in MUN, which in turn is largely influenced by feed intake pattern and characteristics of urea transfer from blood plasma to milk and vice versa. There are, however, few quantitative data available on the exchange of urea between milk and blood plasma. This study aimed to obtain insight in urea transfer characteristics within the mammary gland and from the mammary gland to blood plasma in dairy cows at various levels of PUN and MUN. Urea transfer from milk to blood plasma and urea transfer within the mammary gland itself was tested in a 4 × 4 Latin square design using four lactating cows. Treatments consisted of 4 primed continuous intravenous urea infusions of 0 g/h , 5 g/h, 10 g/h, and 15 g urea /h (primed with 0, 30, 60 and 90 g urea, respectively, at 06:00 h). Boluses of [^{15}N^{15}N]urea were injected in cistern milk at 20, 60, and 100 min before the 17:00 h milking. Milk was collected in portions of approximately 2 L at the 17:00 h milking. Milk samples were analyzed on urea and enrichment of ^{15}N-urea. Increasing urea infusion rate increased milk urea-N concentration from 9.7 to 22.1 mg N/dL. Fractional ^{15}N-urea disappearance rate from milk to blood (K_{urea}) varied between 0.425 and 0.666 /h but was not affected by urea infusion rate nor related to MUN. Cistern injected [^{15}N^{15}N]urea diffused within 20 min after injection toward alveoli milk. Calculations with the K_{urea} observed in this study show that an equilibrium between urea concentration in blood plasma and milk is reached after approximately 9 h, and that K_{urea} can be used in calculating MUN. It is concluded that urea disappearance from milk in the mammary gland is substantial as well as the intra-mammary urea exchange between cistern, duct, and alveoli milk. Information on K_{urea} is useful to quantify the effects of diurnal variation in PUN on MUN, which enhances the utility of MUN as an indicator for N-excretion in urine.

Key words: milk urea, urea transfer, mammary gland, dairy cattle

Introduction

A positive relationship exists between the concentration of urea nitrogen in milk (MUN; mg N/dL) and blood plasma (PUN; mg N/dL) and the excretion of nitrogen (N) in urine (Ciszuk and Gebregziabher, 1994). Because of this positive relationship, MUN is proposed as an estimator for urinary N excretion and ammonia emission (Nousiainen et al., 2004; Burgos et al., 2007; Van Duinkerken et al., 2011a). However, the relationship between MUN and urinary N excretion is affected, among others, by diurnal dynamics in MUN, which in turn is largely influenced by feed intake pattern (Gustafsson and Palmquist, 1993) and characteristics of urea transfer between blood and milk (Spek et al., 2012a). There is a substantial number of studies that have measured the effect of feed or N-intake on diurnal patterns of rumen ammonia (e.g. Gustafsson and Palmquist, 1993; Reynal and Broderick, 2005; Boucher et al., 2007; Agle et al., 2010) and PUN (e.g. Rodriguez et al., 1997; Piccione et al., 2006; Cummins et al., 2009; Røjen et al., 2011). However, the relationship between PUN and MUN has received less attention. Some studies have measured disappearance of injected labeled urea in the mammary gland of milk goats (Linzell and Peaker, 1971) and of dairy cattle (Spek et al., 2012a). However, these studies were small in scale and more information is required on sources of variation before solid conclusions can be drawn. This study aimed to obtain further insight in urea transfer characteristics within the mammary gland and from mammary gland to blood plasma in dairy cows at various levels of PUN and MUN by means of continuous intravenous infusions of urea and by intra-mammary injected pulse doses of [$^{15}N^{15}N$] urea at various times before milking.

Materials and Methods

The study was approved by the Institutional Animal Care and Use Committee of the Animal Sciences Group, Wageningen University and Research Centre, Lelystad, the Netherlands.

Cows, housing, and experimental design

Four lactating Holstein-Friesian cows were selected based on days in milk and milk production. At the start of the experiment, the average body weight of the cows was 636±42.7 kg, parity 2.5±1.00, DIM 90±3.9 d, and milk production 39.8±4.70 kg/d (values expressed as means±SD). Cows were housed in a tie-stall on rubber mats and wood shavings. Cows were randomly assigned to a treatment according to a 4 × 4 Latin square design. Treatments consisted of four levels of continuous intravenous urea infusions (0, 5, 10, and 15 g urea/h) dissolved in a saline solution administered during sample collection days. The experiment lasted 7 weeks and consisted of 3 adaptation weeks to a simultaneous change in housing condition (from free stall to tie-stall) and a change from a high protein diet (~ 160 g CP/ kg DM) to the low protein TMR of 135 g CP/kg DM (Table 1), followed by 4 experimental periods. Each experimental period of 1 week consisted of 3 sample collection days (Monday, Wednesday, and Friday). The days in between sample collection days were included as

washout days for infused urea and injected boluses of labeled urea. Cows had ad libitum access to feed during the first 16 days of the 3 week adaptation period and subsequently feed intake was restricted to 95% of ad libitum feed intake of each individual cow during the rest of the experiment. Cows were milked twice daily at 05:00 h and 17:00 h throughout the experiment. During the non-collection days, cows were fed 2 equal meals, twice daily at 05:00 h and 17:00 h, whereas during collection days 50% of the daily feed allowance was provided in 6 equal meals every 2 h from 05:00 till 17:00 h to minimize variation in PUN and MUN caused by variation in feed intake during that day. At 17:00 h, the remaining 50% of the total daily feed allowance was provided. Daily individual feed intake in the tie-stall was determined by subtracting the fresh weight of orts from the weight of fresh feed supplied.

Infusions and sample collection

Three batches of the TMR rations were made during the experiment and each batch was immediately stored at -20 °C. Two days prior to feeding, the quantity of feed consumed per day was taken out of the freezer to defrost and the average outside temperature (measured at Lelystad airport) during the study period was 6.1°C. From each TMR batch a representative sample (~700 g) was taken immediately after mixing and stored at −20°C. During the third week of the experiment cows were fitted with 3-way blood sampling catheters (BD Careflow triple lumen; Becton Dickinson BV, Breda, the Netherlands) in the jugular vein, and catheters were kept open by a 33 U of heparin/mL saline solution throughout the experiment. The distal and proximal ports of the blood sampling catheters were used for the continuous infusion of urea solutions and the collection of blood samples, respectively during collection days. During collection days, continuous infusions were given (37.5 mL/h) of solutions containing either 0, 133, 267, or 400 g urea/L saline via 50 mL syringe pumps (Perfusor Secura FT, B. Braun, Melsungen, Germany), starting at 06:00 h and ending at 17:00 h. Just before starting infusions cows were primed with an intravenous injection of 0, 30, 60, and 90 g urea (dissolved in saline) for the 0, 133, 267, and 400 g urea/L infusion treatments, respectively. During collection days a bolus of [^{15}N^{15}N]urea was injected in cistern milk at 20 min before the 17:00 h milking on Monday, 60 min before the 17:00 h milking on Wednesday, and at 100 min before the 17:00 h milking at Friday. The [^{15}N^{15}N]urea boluses were injected in the four cisterns of the cow via the teat canals by using a crop needle attached to a 10 mL syringe. The teats and crop needle were disinfected before injection of the boluses by spraying with a 70% ethanol solution before injection in order to minimize the risk of mastitis. Weights of the boluses injected in the cisterns of the mammary gland for the 0, 5, 10, and 15 g urea/h infusion treatments were 6.0±0.13, 11.9±0.08, 17.6±0.27, and 23.6±0.71 mg of [^{15}N^{15}N]urea dissolved in saline, respectively. During collection days milk was collected in portions of approximately 2 L using a WB HI / Pullout Tru-tester device (Tru-Test Ltd., Auckland, New Zealand) at the 17:00 h milking and each portion was sampled (10 mL per sample). Directly after milking, 20 IU of oxytocin was injected intravenously after which the residual milk was

collected and sampled as well (10 mL per sample). Total milk production during the 17:00 h milking was recorded and a representative sample of the total collected milk was composed (10 ml per sample). A blood sample (10 mL) was taken at 3 h before the 17:00 h milking and directly put in ice water. The blood sample was centrifuged at 3,000 × *g* for 15 min at room temperature and blood plasma separated. Blood plasma and milk samples were stored at -20 °C pending analysis. During sample collection days (05:00 – 17:00 h) water intake for each cow was recorded every two hours and also total daily drink water intake from 05:00 h until 05:00 h the next day was recorded.

Analytical procedures

Dry matter content of TMR was determined by over drying at 70 °C during 24 h. Analysis of CP, ash, NDF, ADF, starch, crude fat, Na, K, and Ca was carried out as described by Spek et al. (2012b). Concentrations of fat, protein, lactose, and somatic cell count in milk were analyzed as described by Abrahamse et al. (2008). Milk urea content was determined using the pH difference technique (ISO 14637; ISO, 2004). Concentrations in milk of urea, fat, protein, lactose, and somatic cell count were analyzed on milk samples taken from the total quantity of milk collected during the 17:00 h milking. Milk samples were defatted and deproteinized by centrifugation at 10,600 × *g* after precipitation of protein and fat with sulfosalicylic acid (15%) on a 3:1 (v/v) milk to acid ratio. The resulting fat- and protein-free solution was used for analysis of ^{15}N-urea enrichment by EA-IRMS (EA type DP 200 Series 2, IRMS type Delta S) according to the procedure described by Spek et al. (2012a). For each cow during each sample collection day, a blood plasma sample taken at 3 h before the 17:00 h milking was analyzed on ^{15}N-urea enrichment in the same manner as analysis of ^{15}N-urea enrichment in milk samples in order to determine the estimation of background enrichment of ^{15}N-urea in milk.

Table 1. Dietary composition (g/kg DM unless otherwise stated) of the TMR

	TMR
Ingredients	
Corn silage[1]	668
Wheat straw, chopped	26
Rape meal	160
Soybean hulls	90
Palm fatty acids	20
Limestone	10
Sodium carbonate	10
Molasses	6.9
Urea	2.8
Mineral premix[2]	2.3
Feed salt[3]	1.9
Magnesium sulphate	1.8
Magnesium oxide	0.6
Nutrients	
DM (g/kg feed)	491
CP	135
Ash	66.9
Crude fat	45.7
Starch	235
NDF	368
ADF	236
Ca	7.9
K	9.6
Na	3.8
Feeding value	
NE_L[4] (MJ/kg DM)	6.69
OEB[5]	10
Rumen degradable CP[6]	85

[1]Corn silage (g/kg DM unless specified otherwise): DM, 416 g/kg; CP, 69; starch, 381; NDF, 353; ADF, 196; ADL, 20; ash, 42 (determined with near infra-red spectrometry; Blgg, Wageningen, the Netherlands).

[2]Contained per kilogram of mix: 108 g Ca; 240 g Mg; 4,960 mg Cu; 9,624 mg of Mn; 13,440 mg Zn; 696 mg I; 520 mg Co; 102 mg Se; 2.000,000 IU vit. A; 440,000 IU vit. D3; 6,000 IU vit. E.

[3]Composition of feed salt: ≥99.8% NaCl.

[4]Net energy for lactation calculated with VEM (feed unit lactation) system (Van Es, 1975).

[5]Rumen degraded protein balance (Van Duinkerken et al., 2011b).

[6]Based on Dutch protein evaluation system (Van Duinkerken et al. (2011b).

Statistics

Effects of urea infusion rate on milk yield, milk composition, and water intake were analyzed using a mixed linear model (employing the MIXED procedure of SAS version 9.2), with period and urea infusion rate included as fixed class effects and cow included as random effect. Disappearance rate of ^{15}N-urea from the milk (K_{urea}) was estimated by using the NLIN procedure of SAS (version 9.2) and the following model:

Retained ^{15}N-urea (time) (%) = A (%) × exp(-K_{urea} (/h) × time (h))

Where A is the estimated percentage of recovered ^{15}N-urea at the time of injection of [^{15}N^{15}N]urea in the cisterns of the mammary gland and K_{urea} the disappearance rate of injected [^{15}N^{15}N]urea from the milk in /h.
The [^{15}N^{15}N]urea disappearance characteristics A and K_{urea} were estimated separately for each individual cow for each treatment. Effects of urea infusion rate on the individual estimated A and K_{urea} were analyzed using the MIXED procedure of SAS with period and urea infusion rate included as fixed class effects and cow included as random effect. For all mixed models, 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.

Results
Cows remained healthy throughout the experiment and no cases of clinical mastitis were observed. Dry matter intake, milk production, daily water intake, water intake during infusion, and concentrations of fat, protein, and lactose in milk did not differ significantly between treatments (Table 2). There was a significant effect of urea infusion level on somatic cell count (SCC), being higher for the 0 g/h urea infusion rate compared to the 5 – 15 g/h urea infusion rate treatments (Table 2).

Table 2. Least square estimates of DMI, water intake, urea concentrations in plasma and milk, and milk concentration of fat, protein, lactose and somatic cell count (SCC) for the various urea infusion rates during sample collection days.

	Urea infusion rate (g/h)					
	0	**5**	**10**	**15**	**SE**	**P-value**
DMI (kg/d)	23.0	22.9	23.5	23.2	0.88	0.244
Water intake (kg/d)	80.3	84.7	86.5	82.3	4.75	0.558
Water intake day (kg/12 h)	40.0	39.8	43.9	42.0	1.79	0.205
Milk yield (kg/d)	32.7	32.8	33.4	32.9	2.17	0.476
FPCM (kg/d)	36.5	35.7	37.2	36.8	1.82	0.259
Milk urea N (mg N/dL)	9.7^{a}	14.4^{b}	17.7^{c}	22.1^{d}	1.15	<0.001
Milk fat (%)	5.01	4.77	5.02	5.04	0.342	0.357
Milk protein (%)	3.37	3.39	3.39	3.40	0.115	0.815
Milk lactose (%)	4.65	4.63	4.66	4.65	0.059	0.697
SCC (× 1000/mL)	336^{a}	109^{b}	100^{b}	100^{b}	69.3	0.011

^{a,b,c,d} Within a row least square means without a common intercept differ (P<0.05).

Urea concentration in milk was positively related to urea infusion level (Table 2). Results on ^{15}N enrichment data from one cow receiving the 5 g urea/h infusion treatment were discarded due to leakage of injected ^{15}N labeled urea out of the teats resulting in a K_{urea} value (1.457 /h)

which deviated more than 3 standard deviations from the average K_{urea} value of 0.623±0.2728 of all (n=16) K_{urea} values estimated per cow per treatment. Figure 1 illustrates the observed disappearance of ^{15}N in time and a fitted fractional disappearance rate K_{urea} for the pooled dataset of 0.560±0.0587 (/h) which is 10% lower than average K_{urea} from statistical analysis.
Fractional disappearance rate of injected [$^{15}N^{15}N$]urea from the mammary gland for the various urea infusion treatments were not significantly affected by urea infusion rate and varied between 0.425 and 0.666 /h (Table 3). An estimate of the percentage of injected [$^{15}N^{15}N$]urea recovered from milk at the time of injection varied between 58.2 to 71.2% (Table 3) meaning that a substantial portion of injected [$^{15}N^{15}N$]urea, varying between 28.8 and 41.8%, was not accounted for by collected milk. Cistern injected [$^{15}N^{15}N$]urea rapidly diffused within 20 min after injection toward alveoli milk (Fig. 2).

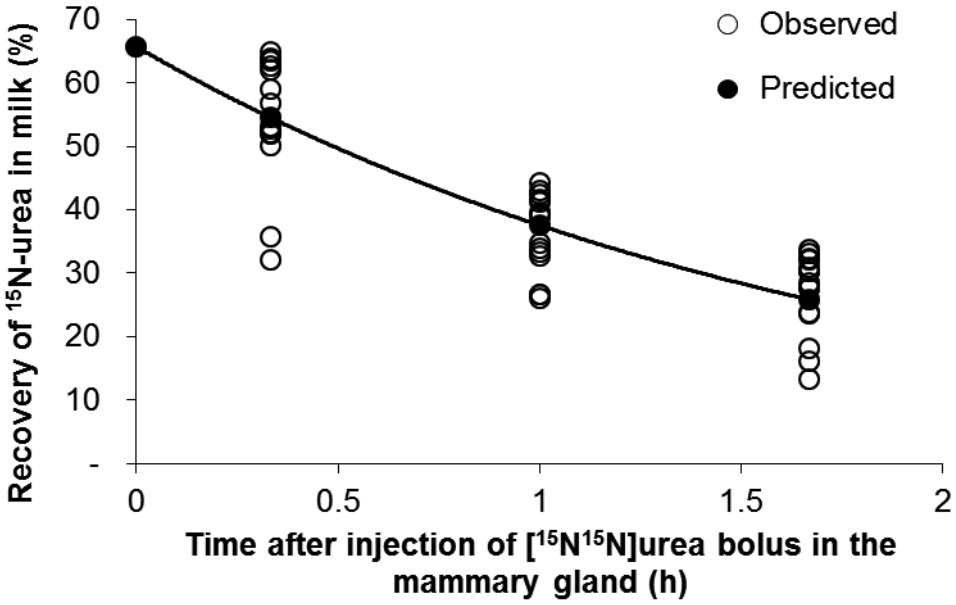

Figure 1. Recovery at milking of [$^{15}N^{15}N$]urea in the mammary gland via the teats at 20, 60, and 100 min after injection. The estimated parameters for the exponential model based on the pooled dataset were: A = 65.7±3.25 (%), K_{urea} = 0.560±0.0587 (/h) (R^2 = 0.71).

Discussion

Urea infusion, MUN, and water intake

Restricted feeding resulted in similar DMI between treatments. Urea infusion rate had no effect on milk production and milk composition except for urea and SCC. Despite the significance reached for SCC, a causal relationship between urea infusion level and SCC has to be excluded. The high SCC for the 0 g/h urea infusion treatment has to be ascribed to one cow that had high SCC levels during the period of receiving the 0 g/h urea treatment whereas SCC levels for that cow during the other treatments were low.

The continuous intravenous infusion of 37.5 mL/h of urea solution containing 400, 267, 133, or 0 g urea/L saline representing urea infusion rates of 15, 10, 5, or 0 g urea/h, respectively, resulted in a wide range in MUN values between 9.7 and 22.1 mg N/dL (Table 2). A positive relationship between urea infusion rate and drink water intake may be expected as a number of studies with dairy cattle show increased urine production with increased N-intake and N-excretion (Broderick, 2003; Colmenero and Broderick, 2006). This increase in urine production (and drink water intake) upon an increase in dietary N-intake can, from a physiological point of view, be explained by increased plasma concentrations of the antidiuretic hormone resulting in an increased glomerular filtration rate and urine production (Bankir and Kriz, 1995). Although the numerical values were higher during the infusion period for the higher two urea infusion rates (Table 2), there was no significant effect of urea infusion level on drink water intake. This might indicate that GFR is not as much affected by urea excretion but by other substances such as cations that are associated with dietary protein intake and increased cation levels in plasma.

Table 3. Least square estimates of disappearance characteristics of injected [$^{15}N^{15}N$]urea in the mammary gland for the various urea infusion in venous blood rates during sample collection days (n = 15).

	Urea infusion rate in venous blood (g/h)					
	0	**5**	**10**	**15**	**SE**	**P-value**
A[1] (%)	58.2	65.6	71.2	66.3	7.13	0.231
K_{urea}[2] (/h)	0.425	0.545	0.666	0.632	0.0800	0.150

[1]A is the model estimated percentage of recovered ^{15}N-urea at the time of injection of [$^{15}N^{15}N$]urea in the cisterns of the mammary gland.

[2]K_{urea} is the fractional disappearance rate of injected [$^{15}N^{15}N$]urea from the mammary gland.

Urea disappearance from the mammary gland

The K_{urea} was not significantly affected by urea concentration in the milk and varied between 0.425 and 0.666 /h. In the study from Spek et al. (2012a) similar urea disappearance rates from the mammary gland were observed of 0.456 and 0.576 /h. There seemed to be a positive linear relationship between MUN and K_{urea} (Table). However, mixed model regression with cow as random effect, period as fixed effect, and MUN included as a fixed continuous independent variable did not result in a significant relationship between MUN and K_{urea} (P=0.079). The absence of a clear relationship between urea concentrations in milk and K_{urea} suggests that the urea flux from milk to plasma is driven by passive diffusion processes and it seems not to be facilitated by urea transporters as is the case in the kidney and gastro intestinal wall. Previous results from Spek et al. (2012a) indicated a negative relationship

between MUN and K_{urea}. However, such a relationship did not become apparent in the present study. The estimated percentage of retained ^{15}N-urea at the time of injection (A) was 65.7±3.25 (%) for the pooled dataset (Fig. 1). This is lower than the A value of 83.5 % established in the previous study of Spek et al. (2012a). The background of 34.3% of injected [$^{15}N^{15}N$]urea not being accounted for is unclear. Several factors may have contributed to this unaccounted fraction of injected [$^{15}N^{15}N$]urea. One possibility is that the milking process did not remove all the milk from the mammary gland. Another possibility is leakage of milk from the teats after injecting the [$^{15}N^{15}N$]urea or just before milking. However, this leakage of milk has been carefully monitored during the trial and was minor. A further possibility is the erroneous assumption that natural enrichment of ^{15}N-urea in milk is equal to that in blood plasma. If natural enrichment of ^{15}N-urea in milk is lower than in blood plasma, this difference in natural enrichment might explain the low estimated percentage of retained ^{15}N-urea at the time of injection. Further, the presence of a lag time might affect the estimate of recovered injected [$^{15}N^{15}N$]urea at the time of injection because some time is required for the injected [$^{15}N^{15}N$]urea to become injected (1 to 3 min) whereas the time of injection was defined as the time the injection procedure started. Another possibility for a lag time is the time required for labeled urea to be distributed throughout the entire milk distribution volume. Finally, the single exponential model representing mass action flow from a single milk compartment may not be representative for dynamics of disappearance of milk urea when more than one compartment or process is involved. Underestimation of the percentage of ^{15}N-urea cleared at 20 and 100 min after injection because of a faster initial disappearance would lead to an underestimation of A (and K_{urea}).

Urea diffusion within the mammary gland

The distribution pattern of [$^{15}N^{15}N$]urea within the mammary gland in time as shown in Fig. 2 is similar to that established in a previous study of Spek et al. (2012a) showing a curve which can be described by 3rd order polynomial. Within 20 min after injection of [$^{15}N^{15}N$]urea in the cisterns, the ^{15}N-urea was homogenous distributed throughout the milk present in the milk fraction 0.2 to 0.9 (Fig. 2). This rapid distribution of [$^{15}N^{15}N$]urea injected in the cisterns towards alveoli milk seems in support of the assumption of a single compartment for milk in the mammary gland. However, the finding of higher ^{15}N-urea enrichment values between milk fraction 0.5 and 0.8 (milk likely originating from the ducts and alveoli) compared to the fraction between 0.4 and 0.5 is actually an indication of the existence of multiple milk compartments.

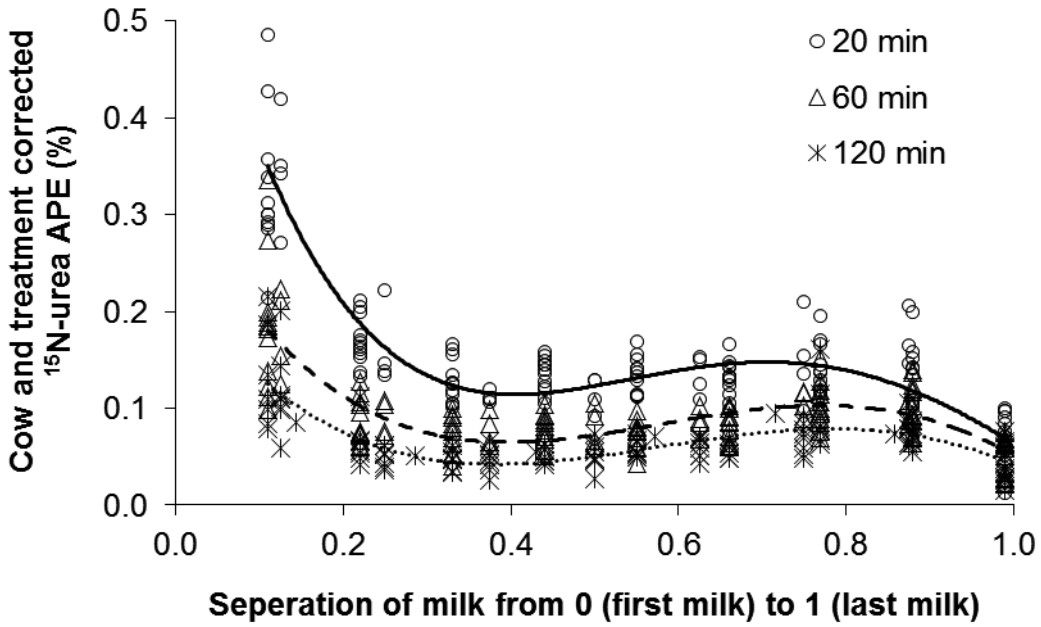

Figure 2. Cow and treatment corrected ^{15}N-urea atom percentage excess (APE) in fractions of milk ranging from first milk to last milk after injecting a [^{15}N^{15}N]urea bolus in the mammary gland at 20, 60, and 120 min before milking. Third order polynomial models were fitted to the pooled data at 20, 60, and 120 min (solid curve, 20 min; dashed curve, 60 min; dotted curve, 120 min).

Modeling urea fluxes between plasma and milk

Because no significant effect of MUN on the fractional urea disappearance rate from milk in the mammary gland was observed (Table 3) it seems likely that the process of simple diffusion explains the transfer of urea towards blood plasma. This transfer is likely determined by factors that affect movement of urea across the membranes of capillaries and udder cells, and likely involves factors such as surface area of exchange between plasma and milk, blood flow, and the speed of which urea molecules move across membranes between plasma and milk. Nevertheless, it seems that the K_{urea} can be regarded as a constant value which accounts for the effect of these factors.

Based on the pooled data set of this study, the K_{urea} was estimated to be 0.560 /h (Fig. 1). Assuming this fractional rate applies to the dynamics of urea transfer from blood plasma to milk, and vice versa, it can be calculated what time period is required for urea-N concentrations in plasma and milk to reach equilibrium after PUN is increased relative to MUN due to protein intake. The assumption was made of a volume of milk and body distribution volume of urea of 15 and 325 L, respectively, an initial MUN of 9.32 mg N/dL, a constant blood plasma urea-N concentration of 13.98 mg N/dL, and a K_{urea} of 0.560 /h. It was furthermore assumed that when PUN and MUN are in equilibrium the absolute flux of urea from milk to plasma is equal to the urea flux of plasma to milk. Because of the larger pool of urea in the plasma the fractional urea disappearance rate of urea from plasma to milk was

calculated as 15 L (urea distribution volume of milk) / 325 L (body urea distribution volume) × 0.560 = 0.0258 /h. The following equations were used to calculate the time period that is required for urea-N concentrations in plasma and milk to reach equilibrium after a 3.5 mg N/dL increase in PUN relative to MUN observed in the study of Gustafsson and Palmquist (1993) for one cow:

Urea flow from milk to blood (g urea-N/h) = Volume of milk (L) × urea-N concentration of milk (g N/L) × fractional urea-N disappearance rate (/h) [Eq. 1]

Urea flow from plasma to milk (g urea-N/h) = Volume of blood plasma (L) × urea-N concentration of plasma (g N/L) × fractional urea-N disappearance rate to milk [Eq. 2]

Urea-N pool in milk (g urea-N) = initial urea N pool in milk (g urea-N) – urea flow from milk to blood + Urea flow from plasma to milk [Eq. 3]

Calculations indicate that after approximately 9 h an equilibrium is reached between urea in blood plasma and milk (Fig. 3).

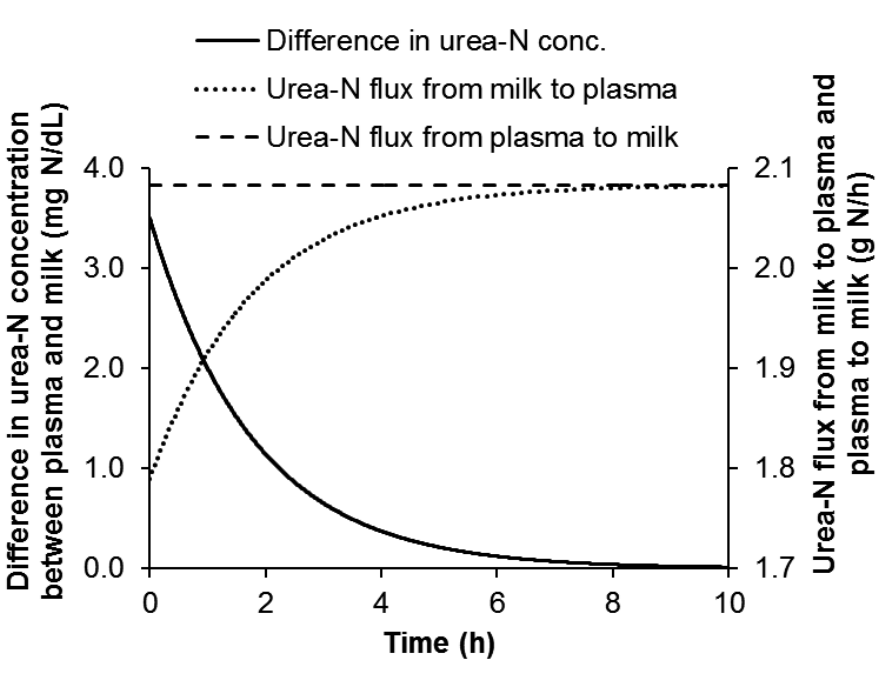

Figure 3. Calculation of the time (h) required to reach an equilibrium between urea-N concentration in milk and blood after an initial difference between plasma and milk of 3.5 mg N/dL, and a constant urea-N concentration based on the modeling assumptions described in the section *Modeling urea fluxes between plasma and milk*. Solid line indicates the concentration difference between urea-N concentration in blood plasma and milk (mg N/dL), dotted line indicates the urea-N flux from milk to plasma (g N/h), and dashed line indicates the urea-N flux from plasma to milk (g N/h).

This is about nine to five times longer than the 1 to 2 h mentioned by Gustafsson and Palmquist (1993). Gustafsson and Palmquist (1993) measured the time course of the urea concentration in blood plasma and milk after a meal, and their results can be used to evaluate the urea dynamics established in the present study. The graphically reported PUN and MUN values in the study of Gustafsson and Palmquist for one cow were translated back to PUN and MUN values and used in the present evaluation. Calculations were performed under assumption of recorded volume of afternoon milk of 12.9 L, and PUN values that were observed after feeding in the study of Gustafsson and Palmquist (1993) were used as inputs. A urea distribution volume in the body of 328 L (50% of reported body weight) was used. The model was run iteratively for 12 h with time steps of 0.001 h. Values of PUN in time between observed PUN values were estimated via linear interpolation. Predicted MUN matched closely with observed development in PUN and MUN after feeding (Fig. 4), which indicates that the K_{urea} values established in the present study are realistic for the testing conditions of Gustafsson and Palmquist (1993) as well. The calculations show that diurnal variation in MUN can be calculated from (expected) diurnal variation in PUN according to the current approach. Such calculations are required to be able to interpret the effect of feed intake patterns on diurnal variation in PUN, and subsequently on MUN. A better understanding of the effect of diurnal variation in PUN on MUN increases the utility for MUN as an indicator for N excretion in urine.

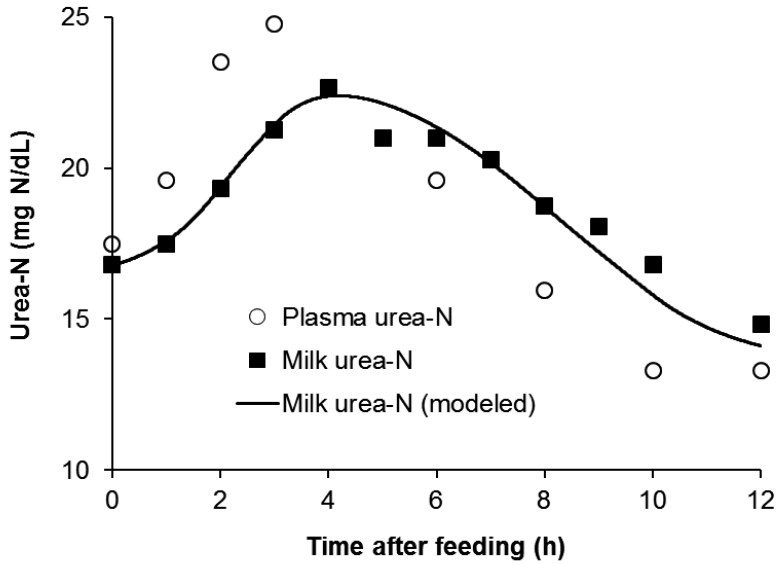

Figure 4. Development of urea-N concentration in blood plasma (open circles) and milk (closed squares) in time (h) after feeding reported by Gustafsson and Palmquist (1993), and the development of calculated urea-N concentrations in milk using the calculations and assumptions such as a urea distribution volume of 12.9 and 328 L in milk and plasma, respectively, and a fractional urea disappearance rate from milk to plasma of 0.560 /h and from blood plasma to milk of 0.022 /h described in the section *Modeling urea fluxes between plasma and milk*.

Conclusions

It is concluded that urea disappearance from milk in the mammary gland is substantial as well as intra-mammary urea transport between cistern, duct, and alveoli milk. Information on the fractional disappearance rate of urea from the mammary gland can be used to quantify the effect of diurnal variation in PUN on variation in MUN. This information enhances the utility of MUN as an indicator for N excretion in urine with different feeding regimes and production conditions.

Acknowledgements

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

Chapter 8

General discussion

Introduction

Milk urea nitrogen (MUN) concentration in dairy cows may serve as an on-farm indicator to guide nutritional strategies and to help reduce emissions of nitrogen (N) to the environment. There is increasing evidence that a significant proportion of variation in MUN is not related to variation in urinary N excretion. The general aim of this thesis was to increase the applicability of milk urea nitrogen (MUN; mg N/dL) as a predictor of urinary nitrogen excretion (UN; g N/d) and urinary urea nitrogen excretion (UUN; g N/d), by identifying and quantifying factors that explain variation in MUN that is not related to variation in UN and UUN. Furthermore, the findings from this thesis will be used in the development of a mathematical model that helps to better interpret the implications of changes in various nutritional and physiological circumstances on MUN. To obtain an overview of factors that may affect the relationship between MUN and UN or UUN, available literature was reviewed (Chapter 2). A number of factors were identified that affect the relationship between MUN and UN or UUN, including dietary crude protein content (CP; % in DM), dietary salt or water intake, diurnal variation in urea entry in blood and milk, bodyweight, breeding value for MUN and biological rhythm.

Moreover, a quantitative meta-analysis was carried out that studied the effect of various physiological and dietary factors on the relationship between MUN and UN or UUN (Chapter 3). It appeared that the combination of MUN and CP could explain much more variation in UN and UUN than either MUN or CP alone. This meta-analysis also showed that attempts to estimate UN assuming zero N balance of the cow, is likely to yield inaccurate and imprecise relationships between MUN and UN. One of the factors found in the literature review that can affect the relationship between MUN and UN or UUN is dietary salt or water intake. In order to quantify the effect of dietary salt on MUN, UN, and UUN an experiment was carried out with lactating cows that investigated the effect of four dietary levels of NaCl on urea levels in blood plasma and milk and on UN and UUN (Chapter 4). The results from this trial clearly showed a negative relationship between dietary NaCl intake and MUN whereas UUN was not affected and UN excretion increased linearly with NaCl intake. The question arose whether the effect of dietary salt on MUN would be similar at high and low dietary protein levels as the renal mechanism of excretion and reabsorption of urea is reported to be affected by both dietary protein and dietary salt intake. Therefore, the interaction between dietary NaCl and protein on UUN was tested in an experiment with lactating cows with two dietary levels of protein combined with a low and a high dietary NaCl level (Chapter 5). No interaction between dietary NaCl and protein on MUN, UUN and renal clearance characteristics was observed. However, the relationship between MUN and UUN was altered by NaCl intake. The literature review (Chapter 2) further revealed that diurnal variation in blood plasma urea N (PUN; mg N/dL) and MUN can be substantial, and that this variation depends on factors such as diet and time and frequency of feeding and milking. Insight into the dynamics of urea transport between blood and milk is important to predict variation in MUN under different

feeding and milking regimes. In a pilot study (Chapter 6) an experimental measurement protocol was tested to establish its applicability to study characteristics of urea transfer from milk to blood and of urea transfer within the mammary gland. In this pilot the disappearance rate of a pulse dose of [$^{15}N^{15}N$]urea in milk and the time of appearance in blood after injection was studied. In the main study (Chapter 7) the disappearance of cistern injected (via teat canals) pulse doses of [$^{15}N^{15}N$]urea from milk was studied together with the distribution of cistern injected [$^{15}N^{15}N$]urea throughout the mammary gland by studying subsequent fractions of milk separated during milking. Information on disappearance characteristics of urea from milk to blood is useful information to model diurnal variation in MUN resulting in a better interpretation of MUN and the relationship between MUN and UUN.

In Table 1 an overview of factors is given that affect the relationship between MUN and UUN or UN as presented in Chapters 2 through 7 and that will be evaluated further in the present discussion.

Table 1. List of factors that affect the relationship between milk urea nitrogen and urinary urea nitrogen excretion together with the chapters that describe these factors.

Factor	**Chapter**
Dietary protein concentration	2 and 3
Salt or water intake	2, 4 and 5
Body weight	2
Diurnal variation in PUN	2, 6 and 7
N-balance	3

A proper understanding of the role of the kidneys in excreting urea in the urine and recycling of urea from the glomerular filtrate back to the plasma is crucial in understanding the relationship between MUN and UUN. In Fig. 1 a simplified flow scheme is presented with important urea fluxes in the lactating dairy cow.

For example, an increase in the glomerular filtration rate (GFR; L/d)) will lead to a temporary increased flux of urea into the glomerular filtrate (flow 3 in Fig. 1) and urine (flow 5 in Fig. 1) resulting in a permanently reduced level of PUN and MUN and a change in the relationship between MUN and UUN. An increase in the renal recycling ratio of urea (RRR) will lead to a temporary reduction of urea excreted in urine resulting in a permanent increase of PUN and MUN and a change in the relationship between MUN and UUN. It is therefore important to identify and quantify factors that affect GFR and RRR.

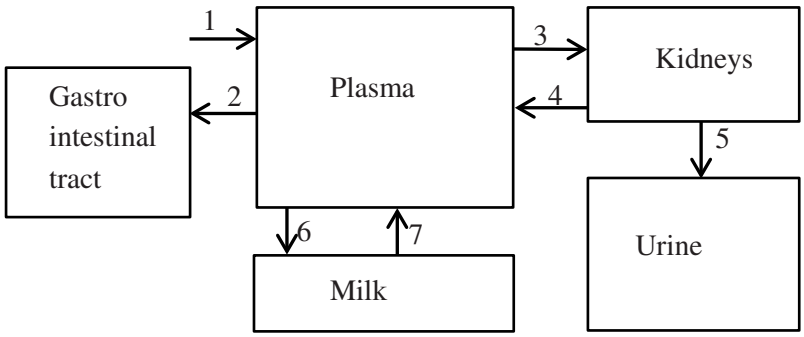

Figure 1. Simplified flow scheme of urea in the lactating dairy cow. Boxes enclosed by solid lines indicate organs or fluids; arrows indicate urea fluxes. Flux 1 represents the flux of urea synthesized by the liver from ammonia coming from the gastro intestinal tract and from oxidized amino acids. Flux 2 represents the urea flux into the gastro intestinal tract. Flux 3 represents the urea flux into the glomerular filtrate. Flux 4 represents the recycling of urea from the glomerular filtrate back to the plasma pool. Flux 5 is the flux of urea in the glomerular filtrate excreted in the urine. Fluxes 6 and 7 represent the transport of urea from the plasma to the milk and vice versa, respectively.

Renal Handling of Urea

Effect of N-intake on GFR and RRR

Results from this thesis (Chapter 5) and other studies with dairy cattle, beef, sheep and goats generally show that dietary N-intake is positively (although not in all cases significant) related to GFR (Fig. 2) and negatively related to RRR (Fig. 3).

The effect of N-intake on RRR and GFR can, from a physiological point of view, be regarded as a way to clear blood plasma from excess urea but also as a means to preserve urea for microbial protein synthesis purposes during times when N-intake is low. The combined response to an increase in N-intake and increase in endogenous urea synthesis by a simultaneous lowering of RRR and increase of GFR corresponds with the increased precision of estimating UN and UUN in Chapter 3 using a multivariate model that includes both MUN and CP compared to a univariate model including either MUN or CP. With an increase in N-intake, UUN increases as well, but plasma urea and MUN increase less than proportional. Despite the positive relationship between N-intake and GFR found across studies (Fig. 2), this relationship remains insignificant within individual studies with dairy cattle such as that of Spek et al. (2013c; Chapter 5, $P=0.168$) and of Røjen et al. (2011; $P=0.15$). However, a meta-analysis of all studies depicted in Fig. 2 in which study was included as a random effect and where GFR and N-intake (g N/d) were expressed per kg body weight (BW) did result in a significant positive relationship ($P<0.001$; slope of 1.46 ± 0.269) between N-intake and GFR, although large differences remained between studies. For most of the studies shown in Fig. 3 a strong negative relationship between N-intake (g N/d) and RRR was observed. A meta-analysis of all studies depicted in Fig. 3 in which study was included as a random effect and

where N-intake was expressed per kg BW did result in a strong negative relationship between N-intake and RRR (P<0.001; slope of -51.0±9.08).

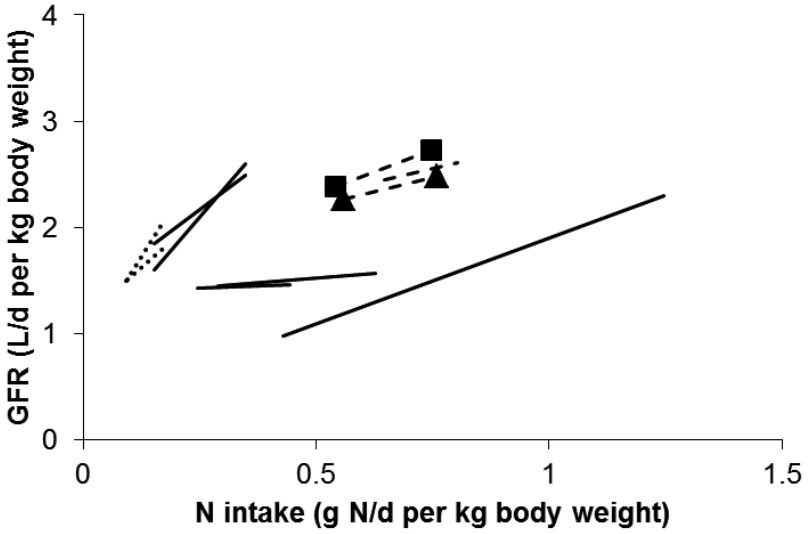

Figure 2. Relationship between dietary N-intake and glomerular filtration rate (GFR) for lactating dairy cattle (Røjen et al., 2011; Spek et al. 2013c (Chapter 5): dashed lines), beef cattle (Thornton, 1970: dotted lines), and for sheep and goats (Ergene and Pickering, 1978; Eriksson and Valtonen, 1982; Van der Walt et al., 1999; Marini et al., 2004: solid lines). Solid squares and triangles are observations from Spek et al. (2013c; Chapter 5).

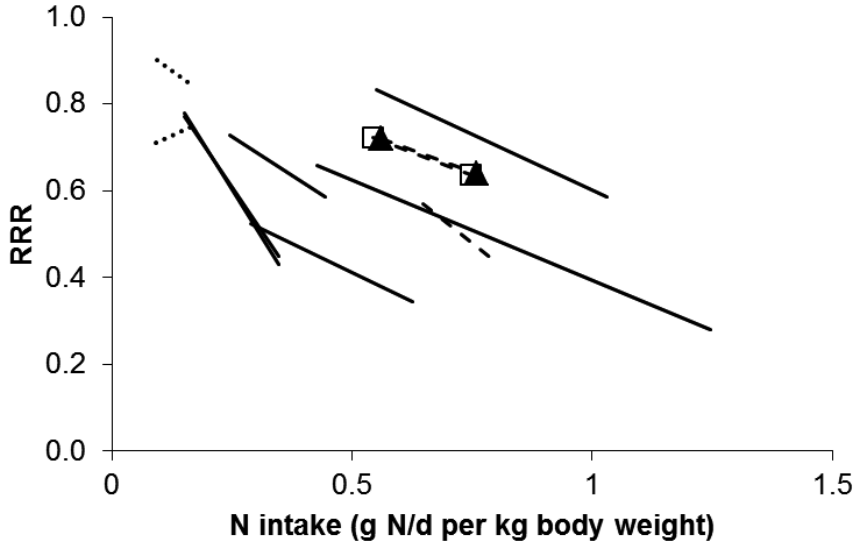

Figure 3. Relationship between dietary N-intake and the renal recycling ratio (RRR) for lactating dairy cattle (Røjen et al., 2011; Spek et al. 2013c (Chapter 5): dashed lines), beef cattle (Thornton, 1970: dotted lines), and for sheep and goats (Ergene and Pickering, 1978; Eriksson and Valtonen, 1982; Van der Walt et al., 1999; Marini et al., 2004; Starke et al., 2012: solid lines). Open squares and solid triangles are observations from Spek et al. (2013c; Chapter 5).

Data from dairy cattle (Røjen et al. (2011) and Spek et al. (2013c; Chapter 5)) showed a similar relation between GFR and N-intake (Fig. 2), but RRR seems to differ substantially more (Fig. 3). This large difference in RRR may be related to the large average positive N-balance observed in the study of Spek et al. (2013c; Chapter 5) of 47 g N/d compared to the low average negative value of -31 g N/d in the study of Røjen et al. (2011). Modelling of observations in section 'Effect of positive N-balance on establishing MUN - UUN relationships' in this chapter suggests that the positive N-balance is best explained by the hypothesis that it consists of urea excreted in urine but for some reason not measured. If this is true than RRR was overestimated in the study of Spek et al. (2013c; Chapter 5) which can explain (at least part of) the differences with the results of Røjen et al. (2011).

Effect of urine production on GFR and RRR

The relationship between urine production and GFR from studies described in this thesis (Chapters 4 and 5) and from studies with beef cattle and sheep are shown in Fig. 4. No significant relationship was observed between urine production and GFR in the studies described in Chapters 4 and 5. However, a meta-analysis of all studies included in Fig.4 in which study was included as a random effect and where GFR and urine production (g/d) were expressed per kg BW, resulted in a significant positive relationship ($P<0.001$, slope of 0.0124 ± 0.00238) between GFR and urine production.

The relationship between urine production (g/d) and RRR from studies described in this thesis (Chapters 4 and 5) and from studies with beef cattle and sheep are shown in Fig. 5 and show that no relationship between urine production and RRR is found for studies with dairy cattle and sheep.

Similar to the relationships established between N-intake and GFR or RRR, even after correcting for BW, large between study differences in GFR (Fig. 4) and RRR (Fig. 5) remained for this set of studies which included a wide range of urine production rates obtained through varying dietary NaCl content or by restricting drink water intake. The cause of these differences is not clear, but these differences in GFR and RRR may also be responsible for the differences in MUN in response to changes in NaCl intake. For example, per 100 g Na intake/d a decrease in MUN of 2.0 mg N/dL was observed by De Campeneere et al. (2009), of 0.7 mg N/dL by Spek et al. (2012b; Chapter 4), and of 0.9 mg N/dL by Spek et al. (2013c; Chapter 5).

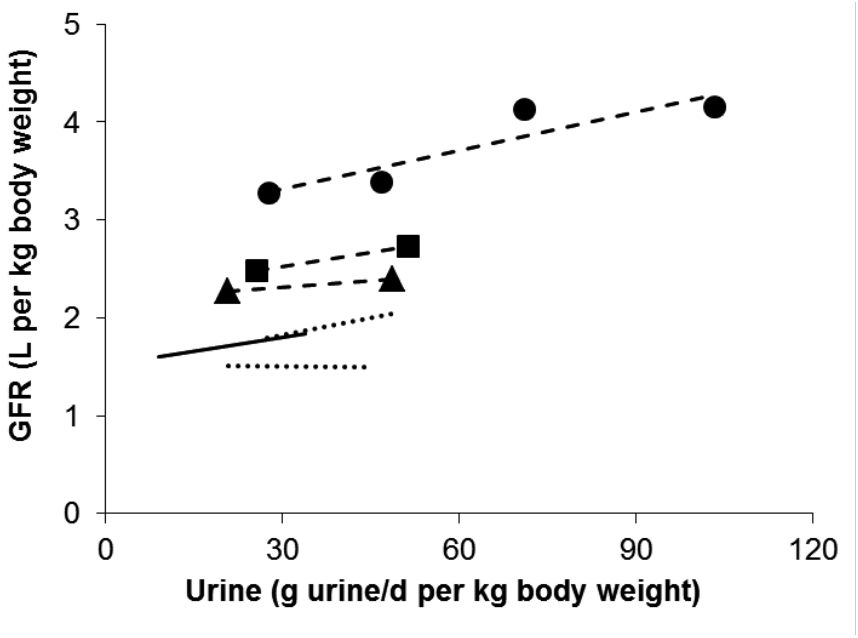

Figure 4. Relationship between urine production and glomerular filtration rate (GFR) for lactating dairy cattle (Spek et al., 2012b (Chapter 4); Spek et al. 2013c (Chapter 5): dashed lines), beef cattle (Thornton, 1970: dotted lines), and for sheep (Ergene and Pickering, 1978; solid line). Solid circles are observation from Spek et al. (2012b; Chapter 4) and solid squares and triangles are observations from Spek et al. (2013c; Chapter 5) where the triangles and squares represent low and high protein treatments, respectively. Variation in urine production within an experiment was obtained by varying drink water intake or salt intake.

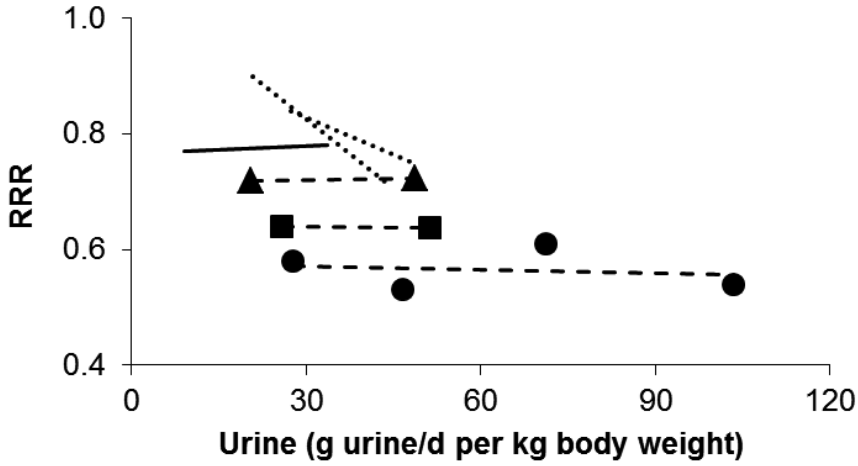

Figure 5. Relationship between urine production affected by salt or drink water intake and renal recycling ratio of urea (RRR) for lactating dairy cattle (Spek et al., 2012b (Chapter 4); Spek et al.c 2013 (Chapter 5): dashed lines), beef cattle (Thornton, 1970: dotted line), and for sheep (Ergene and Pickering, 1978: solid line). Solid circles are observation from Spek et al. (2012b; Chapter 4) and solid squares and triangles are observations from Spek et al. (2013c; Chapter 5) where the triangles and squares represent low and high protein treatments, respectively. Variation in urine production within an experiment was obtained by varying drink water intake or salt intake.

Effect of BW on GFR and RRR

A review of literature (Chapter 2) indicated that the relationship between MUN and UN is likely to be affected by BW. The effect of BW has been observed in experiments with different cattle breeds (Holstein vs. Jersey in the study of Kauffman and St-Pierre, 2001), as well as for data based solely on Holstein Friesians (Kohn et al., 2002; Zhai et al., 2007). A meta-analysis based on sheep, goats, beef cattle, and dairy cattle indicates that this effect of BW on the relationship between MUN and UN or UUN can be explained by GFR as GFR is positively related to BW whereas RRR is not related to BW (Fig. 6).

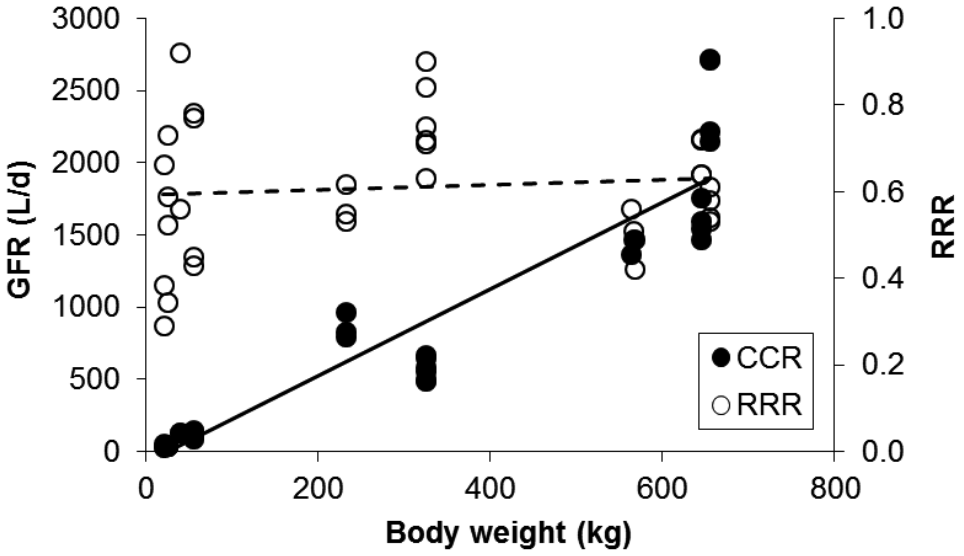

Figure 6. Relationship between body weight and glomerular filtration rate (GFR), and between body weight and renal recycling ratio of urea (RRR). Observations from studies with sheep and goats (Ergene and Pickering, 1978; Eriksson and Valtonen, 1982; Van der Walt et al., 1999; Marini et al., 2004; Starke et al., 2012: body weight less than 200 kg), studies with beef cattle (Weeth and Lesperance, 1965; Thornton, 1970: body weight between 200 and 400 kg), and studies with dairy cattle (Røjen et al., 2011; Spek et al. 2012b (Chapter 4); Spek et al., 2013c (Chapter 5): body weight more than 400 kg). RRR (dashed line) = 0.59±0.042 + 0.000±0.0001 × body weight (kg); R^2 = 0.00. GFR (L/d: solid line) = -80.6±82.76 + 3.01±0.209 × body weight (kg); R^2 = 0.87.

Furthermore, no effect of BW on PUN could be established (P = 0.941, R^2 = 0.00) which supports the conclusion that the effect of BW on the relationship between MUN and UN or UUN across ruminants species is mediated mainly through GFR.

Modelling of MUN

Effect of CP on relationship between MUN and UUN

It was argued in a previous section that the relationship between MUN and UUN is largely determined by GFR and RRR and that GFR and RRR are affected by N-intake. With the aim

to quantify the effect of N-intake or CP content on the relationship between MUN and UUN a static model was constructed under assumption of a constant DM intake of 20 kg/d, 140 g true protein N excreted in milk, a 28 L/d milk production, and 45 g non-urea-urinary-nitrogen (NUUN) excreted per day. Daily N-excretion in feces was set at 170 g N at 13% CP and for every per cent increase in CP above 13% an extra 3 g N/d was assumed to be excreted in feces, i.e., an apparent fecal N digestibility of 90% above base line was assumed. In the model, milk N was assumed to be fixed and unrelated to CP. This assumption does not deviate much from reality as low marginal N-efficiencies are reported between 10 and 20% such as 10% (Huhtanen and Hristov, 2004), 10 – 15% (Huhtanen and Hristov, 2009), and 20% (Kebreab et al., 2010). Moreover, these low marginal protein efficiencies reported in literature might also be partly explained by the positive effect of CP on DM intake resulting in extra energy intake and the probability that mainly energy, and not protein, is the limiting factor for milk protein production. The net plasma urea entry rate (NUER; g N/d) was calculated as N-intake minus N excreted in milk, feces, and as NUUN. Excretion of UUN was calculated as the plasma urea concentration × GFR × (1 – RRR). Furthermore, an empirical relationship for GFR was derived by regression of data from studies with sheep and goats (Ergene and Pickering, 1978; Van der Walt et al., 1999), studies with beef cattle (Thornton, 1970), and studies with dairy cattle (Spek et al., 2012b (Chapter 4); Spek et al., 2013c (Chapter 5)), with study included as random effect and assuming GFR depends on urine production and dietary N-intake:

GFR (L/d/kg BW) = 0.91±0.220 + 1.92±0.389 × N-intake (g N/d/kg BW) + 15.0±4.92 × urine (kg/d /kg BW).

A similar procedure was followed to describe RRR by regression of data from studies with sheep and goats (Van der Walt et al., 1999; Marini et al., 2004; Starke et al), studies with beef cattle (Thornton, 1970), and studies with dairy cattle (Spek et al. 2012b (Chapter 4); Spek et al., 2013c (Chapter 5)), with study as random effect and assuming RRR depends on CP:

RRR = 0.897±0.0604 – 0.0236±0.00411 × CP (% in DM).

In the iterative process to obtain steady state solutions, at every time step the PUN was calculated as the urea pool in the urea distribution volume at the previous time point, plus the sum of incoming urea flux calculated by NUER minus the outgoing urea fluxes to urine and milk divided by the urea distribution volume of the animal which was assumed to be 50% of BW with a cow BW of 650 kg. The initial urea pool in the urea distribution volume was set at 48 g urea-N. The MUN was calculated as a function of PUN and based on the following empirical relationship derived from data that were available from the studies described in chapter 4, 5, and 7:

MUN = -0.76±0.285 + 0.827±0.0201 × PUN (mg N/dL), R^2 = 0.93

Model calculations were performed at two urine production levels (15 and 30 kg/d) and with 1% increments of CP from 13 to 17%. For each CP level and for each urine production level the model was run iteratively for 130 h with time steps of 1 h to achieve steady state with a PUN value that causes inflows of urea in the urea distribution volume to equal outflows of urea in urine and milk.

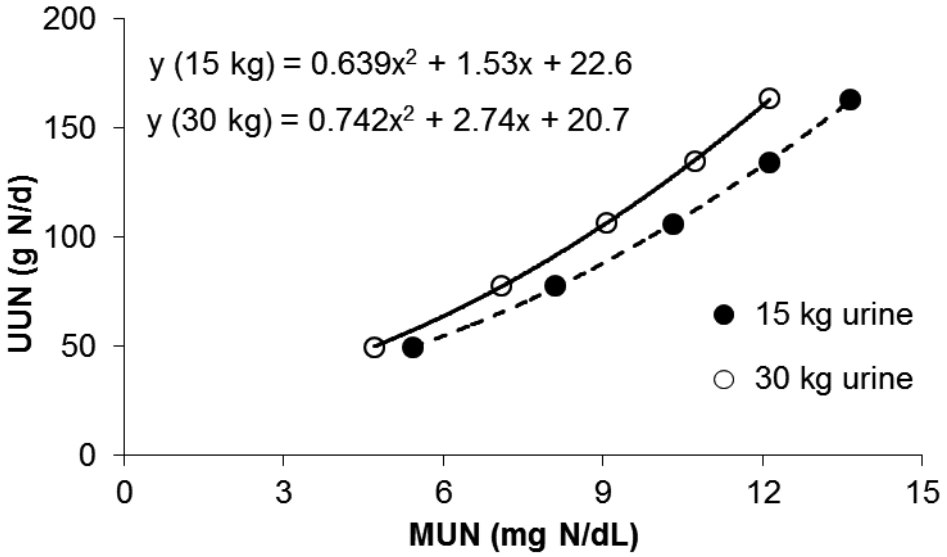

Figure 7. Calculated effect of urine production (15 or 30 kg/d) and dietary protein content (13, 14, 15, 16, and 17 % of dietary DM) on the relationship between milk urea nitrogen concentration (MUN; mg N/dL) and urinary urea nitrogen excretion (UUN; g N/d). Linear regression relationships were as follows: UUN (15 kg urine) = 13.71 × MUN – 29.9; UUN (30 kg urine) = 15.20 × MUN – 26.4.

The results of this modelling exercise are shown in Fig. 7. A quadratic relationship between MUN and UUN is obtained, showing that with an increase of CP the increase in MUN per unit increase of UUN decreases. The quadratic relationship shows that the relationship between MUN and UUN depends on the selected range in CP. This finding is supported by the results from the meta-analysis presented in Chapter 3 indicating that the increase in UUN per unit increase in MUN was lower for the sub-dataset containing lower than average CP observations compared to the sub-dataset containing higher than average CP observations. Furthermore, compared to the simulated MUN values for the 15 kg urine data, the lower simulated MUN values for the 30 kg urine data are in line with the results presented in Chapters 4 and 5.

The regression coefficients for MUN derived from linear regression on the simulated data (equations given in legend of Fig. 7) are in line with regression coefficients for MUN with linear regression relationships established in other studies and hence indicates that the effects of N-intake and urine production on GFR, and the effect of CP on RRR are realistic and help to explain the variation in the relationships between MUN and UUN reported in literature.

Effects of positive N-balance on establishing MUN - UUN relationships

In the modelling exercise presented in the previous section a zero N-balance was assumed. However, usually N-balances observed are positive, as shown in a meta-analysis by Spanghero and Kowalski (1997), and N-balances were positive as well in the studies presented in this thesis (Chapters 3, 4, and 5). Furthermore, it seems that the size of this positive N-balance increases with level of digestible N-intake. Spanghero and Kowalski (1997) pointed out that the size of the average positive N-balance is so large that it cannot be explained as N retained in the animal as body protein. Apparently, part of the excreted N is not accounted for. At present, the origin or form of this unaccounted N remains unclear, but considering the fact that this quantity can be substantial (e.g. as large as 14% of N-intake in Chapter 5), the N unaccounted for requires further investigation. In this section the effect of a positive N-balance on the relationship between MUN and UUN was modelled by using two different approaches using the same model as described in the previous section with few adaptations to account for a positive N-balance. In the first approach (approach A) it was assumed that a positive N-balance is due to urea excreted in urine but for some reason not analyzed as such. In the second approach (approach B) it was assumed that the positive N-balance is due to N-losses other than urea. In approach A the positive N-balance was subtracted from the UUN, whereas in approach B the positive N-balance was subtracted from the calculated NUER. Daily urine production was set at 20 kg/d.

Using the dataset used for the meta-analysis in Chapter 3 with study included as random effect, a positive relationship between dietary CP content and N-balance (P= 0.005) was observed:

N-balance (g N/d) = -25.8±22.29 + 3.6±1.26 × CP (% in DM).

This relationship was used to calculate the size of the positive N-balance with the 1% increments of CP from 13 to 17%. Results of relationships obtained between UUN and MUN are presented in Fig. 8 for the situation where 1) a zero N-balance is assumed; 2) a positive N-balance is assumed with calculations using approach A; 3) a positive N-balance is assumed with calculations using approach B; and 4) the relationship between UUN and MUN was modelled according to the regression equation presented in Chapter 3 (model 14 for EU conditions; UUN predicted from CP and MUN) where CP and corresponding UUN values were taken from the model assuming a zero N-balance, these values were plugged into the

regression equation of model 14 in Chapter 3 in order to derive MUN. Results of these four approaches to model UUN - MUN relationships are presented in Fig. 8.

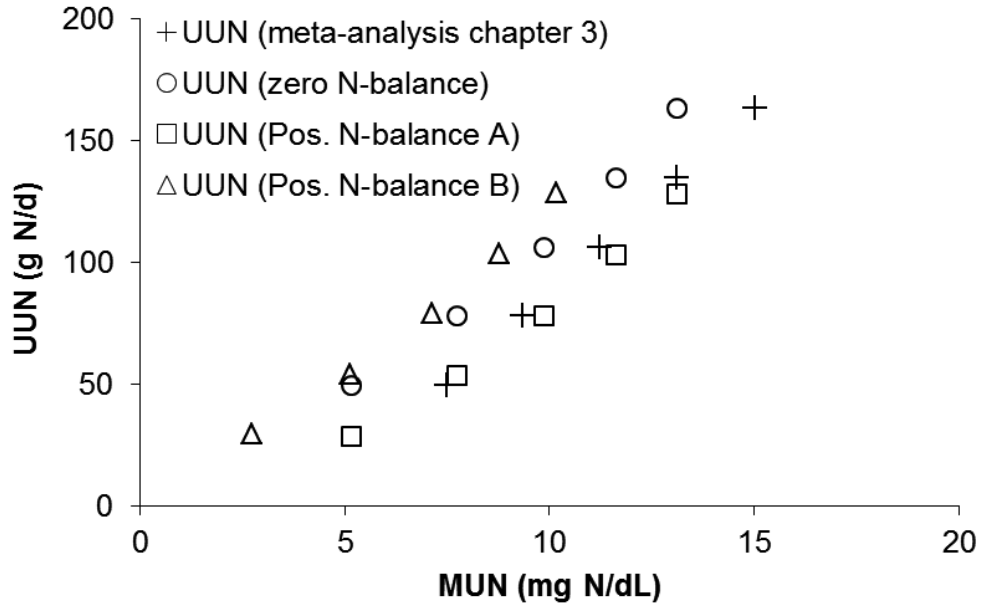

Figure 8. Model calculations on the effect of variation in dietary protein content (13, 14, 15, 16, and 17% of dietary DM) on the relationship between milk urea nitrogen concentration (MUN; mg N/dL) and urinary urea nitrogen excretion (UUN; g N/d)). UUN (meta-analysis chapter 3): same values as for UUN (zero N-balance) at the various CP values, however, the MUN is calculated by inserting CP and UUN values in the regression equation presented in Chapter 3 (model 14) and MUN is calculated from this equation. UUN (zero N-balance): a zero N-balance was assumed. UUN (Pos. N-balance A): a positive N-balance was assumed using approach A. UUN (Pos. N-balance B): a positive N-balance was assumed using approach B.

The results in Fig. 8 for UUN predictions based on model 14 in Chapter 3 were based on in vivo observations and hence include the effect of the observed positive N-balances. The fact that predicted UUN values using approach A (in contrast to approach B and the approach assuming a zero N-balance) are in line with the predicted UUN values using model 14 from Chapter 3 suggests that approach A rightly accounts for the presence of positive N-balances whereas approach B does not. These results indicate that, for an unknown reason, part of the urea excreted in urine was not measured and that therefore the N-balance may have an important effect on MUN – UUN relationships.

Effect of BW on establishing MUN – UN relationships

In a previous section in this chapter it was suggested that the effect of BW on the relationship between MUN and UN or UUN is mediated through GFR. The effect of BW on the relationship between MUN and UN was modelled using an adapted version of the model

described in the previous section '*Effect of CP on relationship between MUN and UUN*' and compared to results from Kauffman and St-Pierre (2001). In this adapted version a number of new assumptions had to be made as dry matter intake (DMI; kg/d) and milk production are affected by BW. The DMI was assumed to be 3.5% of BW (similar to results from Kauffman and St-Pierre (2001)), the NE_L content of the diet was set at 1.66 Mcal/kg DM, the protein and fat concentrations of the milk were fixed at 3.5 and 4.3%, respectively. Furthermore, milk production was calculated based on NRC (2001) net energy for lactation (NE_L) requirements for maintenance (dependent on BW) and milk production (dependent on fat concentration in the milk) with the following formula: milk production (kg/d) = ((DMI × 1.66 Mcal/kg DM) – (0.08 × ($BW^{0.75}$))) / (0.36 + 0.0969 × 4.3%). Furthermore, daily urine production was fixed at 20 and 14 kg/d for a BW of 650 and 450 kg, respectively , NUUN was calculated as 0.069 g N per kg BW/d, and N-excretion of feces was calculated as a function of DMI and CP and based on the following empirical relationship derived from the dataset used for the meta-analysis in Chapter 3:

Fecal N (g N/d) = -68±24.0 + 10.4±0.82 × DMI (kg/d) + 2.0±1.10 × CP (%).

Initial urea pools of the urea distribution volume were 48 and 33 g urea-N for a BW of 650 and 450 kg, respectively. Model calculations were performed by increasing CP with steps of 1% from 13 to 17% for a BW of 450 kg (representing the BW of a Jersey cow) and 650 kg (representing the BW of a Holstein Friesian cow). This CP range from 13 to 17% was also the CP range in the study of Kauffman and St-Pierre (2001). The model was run iteratively for 130 h with time steps of 1 h to reach steady state with inflows of urea in the urea distribution volume equalling urea outflows to urine and milk. The modelled MUN-UN relationships for a 650 kg cow (UN = 17.0 × MUN) and a 450 kg cow (UN = 11.1 × MUN), although slightly lower, were in line with relationships observed by Kauffman and St-Pierre (2001) for Holstein cows (UN = 17.6 × MUN) and Jersey cows (UN = 11.8 × MUN). Kauffman and St-Pierre (2001) noticed that, when UN excretion was expressed per kg of BW, differences in regression coefficients for MUN between Holstein and Jersey cows disappeared. When UN results of this modelling exercise for a 650 kg and a 450 kg cow were expressed per kg BW these differences disappeared as well (Fig. 9). Moreover, when expressing UN per kg of BW, the regression coefficients for MUN for a 650 kg cow (0.0262) and a 450 kg cow (0.0246) match the regression coefficient for MUN established in the study of Kauffman and St-Pierre (2001) of 0.0259 (Fig. 9).

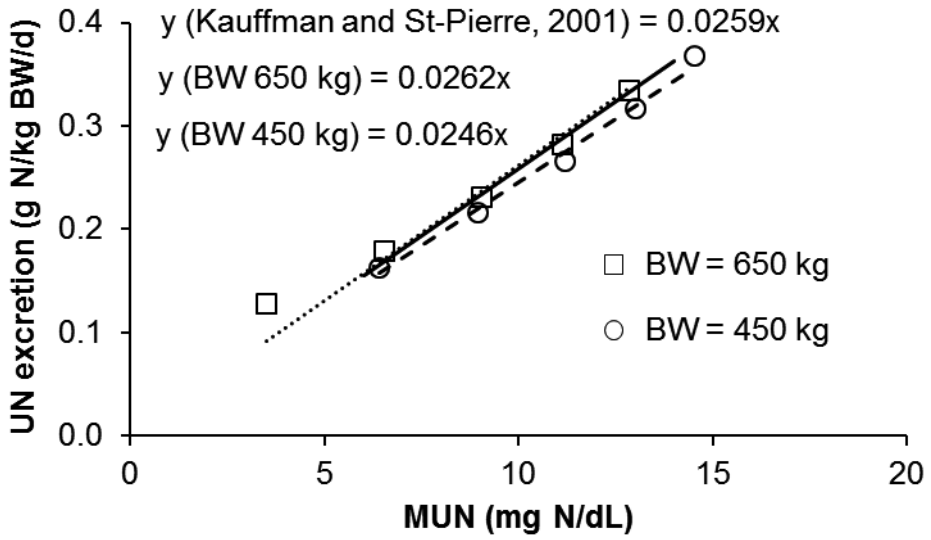

Figure 9. Modelled relationship between urinary nitrogen excretion (UN) per kg of BW and milk urea nitrogen (MUN) for a 650 kg cow (dotted line) and a 450 kg cow (dashed line). The solid line represents the relationship observed by Kauffman and St-Pierre (2001).

In this modelling exercise, GFR was dependent on BW and varied at a CP of 17% from 1.00 L/min for a 450 kg cow to 1.44 L/min for a 650 kg cow. Furthermore, GFR strongly affected MUN as the modelled MUN for a 650 kg cow receiving a CP diet of 17% and having a GFR of 1.44 L/min increased from 12.9 to 18.7 mg N/dL when GFR was lowered to 1.00 L/min (all other parameters remaining the same).

In conclusion, the results of this modelling exercise supports the hypothesis that the effect of BW on MUN-UN relationships is mediated through GFR.

Diurnal Variation in MUN and PUN

A substantial diurnal variation in PUN exists which also affects MUN (Chapter 2), involving the characteristics of urea transfer between plasma and milk. This diurnal variation in PUN and MUN affects the relationship between MUN and UUN and accounts for some of the variation in MUN that is not related to UUN. Being able to predict diurnal variation in PUN and MUN is therefore essential for a correct interpretation of the relationship between MUN and UUN. Diurnal variation in PUN is mainly the result of the diurnal variation in ammonia flux from the rumen to blood as a consequence of the time and frequency of feeding (Chapter 2). In order to quantify the effect of time and frequency of feeding on PUN, MUN, and on the relationship between MUN and UUN, information is required on the characteristics of urea transfer between milk and blood. Chapters 6 and 7 of this thesis provide information to quantify this urea transfer. The modelling results in Chapter 7 show that variation in MUN in time after a meal can be modelled accurately based on the fractional disappearance rate of mammary gland injected ^{15}N-urea and on information on PUN after a meal. Since MUN can

be predicted from PUN with high accuracy (see results in Chapter 7), the question remains on how to predict diurnal variation in PUN. To answer this question, information is needed on incoming fluxes including ammonia fluxes from the rumen to blood plasma and from amino acids catabolized by the animal metabolism. There is a substantial number of studies that have measured the effect of feed or N-intake on diurnal rumen ammonia patterns and/or plasma urea concentrations in time (Gustafsson and Palmquist, 1993; Rodriguez et al., 1997; Reynal and Broderick, 2005; Piccione et al., 2006; Boucher et al., 2007; Cummins et al., 2009; Agle et al., 2010; Røjen et al., 2011) that can be used to model the rumen ammonia flux to blood plasma. Furthermore information is required on diurnal variation in outgoing urea fluxes from the urea distribution volume towards urine and the gastro intestinal tract. However, few studies have investigated the effect of diurnal variation in urea fluxes to urine and the gastro intestinal tract. Clark et al. (2011) observed a clear diurnal variation in urinary N-excretion in dairy cattle housed in metabolism stalls being offered fresh grass two times a day. However, in the study of Clark et al. (2011) PUN was not mentioned and therefore the possible relationship between PUN and diurnal urinary N-excretion could not be tested. More information on factors affecting diurnal excretion of urea in urine such as PUN, intake of minerals, and circadian rhythmicity is required. Because the diurnal variation in PUN and MUN is for the largest part dependent on feed intake patterns the use of mechanistic models seems to be the most appropriate way to predict diurnal variation in PUN and MUN. Furthermore, as shown in Chapter 2, circadian patterns with respect to PUN might be present that are independent of feed intake that may have to be accounted for as well but data on this subject are scarce.

Urea Transport to the Gastro Intestinal Tract

As urea transport from blood to the gastro intestinal tract is a well-established fact (Houpt, 1968, Reynolds and Kristensen, 2008) it is possible that factors affecting this urea transport may also affect PUN and ultimately the relationship between MUN and UUN. Two studies were carried out in this PhD project that investigated the effect of dietary NaCl content on the gastro intestinal entry rate of urea (GER) and one of these studies also investigated the effect of dietary CP content on GER. Results of dietary NaCl and CP from one study are reported in Chapter 5 whereas results from the second study (study described in Chapter 4) are not published. No effect of dietary NaCl content on GER was observed, although GER tended to decrease in one study (P=0.076, Chapter 5) with an increase in dietary NaCl content. When expressed as fraction of UER, GER tended to decrease with increased dietary NaCl content in the other study (P=0.099, unpublished results from the study described in Chapter 4). The GER was significantly increased with higher CP content, and in particular significantly decreased when represented as a fraction of UER (Chapter 5). The possible effect of GER on the relationship between MUN and UUN is not clear as an increase in GER might coincide with an increased ammonia transport from the GIT to blood plasma, resulting in a small or

even no change of net entry rate of ammonia/urea to blood plasma, leaving PUN and MUN rather unchanged. It can even be argued that the urea distribution volume comprises both the volume of blood plasma and the liquid volume in the GIT and that making a distinction between the GIT and blood plasma is arbitrary as long as urea entry does not change microbial synthesis and feed substrate degradation. For example, PUN and rumen NH_3 concentration were positively associated (P=0.033) in the dataset from Chapter 5 and similar relationships have been observed in other studies (Gustafsson and Palmquist, 1993; Kristensen et al., 2010). Urea transfer to the GIT depends on the urea concentration gradient between blood plasma and the rumen (wall) and passive diffusion or facilitated transport (by means of urea transporters) seems to be the principal mechanism with the notification that high rumen NH_3 concentrations appear to negatively affect the transport of urea from blood to rumen (Abdoun et al., 2007; Reynolds and Kristensen, 2008). It is concluded that the main mechanism that actively determines GER as a fraction of UER is the renal recycling mechanism of urea. As RRR increases, more urea is retained in plasma and PUN increases, increasing GER as well. The GER as a fraction of UER may get close to 1.0 when CP becomes smaller than 7.5% (Reynolds and Kristensen, 2008). Labeled urea may be used to determine GER, calculated as the balance difference between UER and the amount of urea excreted in urine and milk. As a consequence of this indirect determination of GER, the presence and magnitude of a positive N-balance might severely affect GER values if this positive N-balance is due to urea excreted in urine which remained unnoticed.

Government Policy on N-emissions and Application of MUN as an Indicator

In the Netherlands, legislation on manure application limits the use of N per ha and calculated N surplus on the farm level based on estimates of N-excretion per animal and number of animals present. At present, in the default situation N-excretion of dairy cattle (including urine and feces) at individual dairy farms is estimated based on the average MUN and milk production per cow per year. The strength of this approach is that both these indicators are easily available as they are already recorded with standard milk control. However, the precision of estimation of N-excretion by these indicators is low, and may deviate substantially from reality due to reasons mentioned in this thesis. Exactly for the reason that making use of the MUN value on itself as an indicator for N-excretion is too inaccurate for specific farming conditions, an alternative method was developed and is currently being applied in practice (called 'BEX') in which the farmer can derive N-excretion in manure from farm specific data on diet and animal productivity. However, this method makes a kind of feed budget account in a retrospective manner on a yearly basis, whereas MUN delivers the farmer information on estimated N-excretion on an almost on-line basis. The MUN values from milk control hence deliver the farmer an easily accessible and fast indicator which offer more opportunity to intervene in nutritional and farm management. The latter is important with the aim to reduce ammonia emissions and to optimize N-utilization by the dairy herd as

best as possible. Results of this thesis show that the usefulness of MUN as an estimator of UN and as a management tool to minimize UN or UUN is increased after correction of MUN for the effect of CP, water intake (as affected by salt intake or type of diet such as maize or grass silage), BW, and diurnal variation of MUN. From the above considerations it is concluded that MUN can be of great interest as a daily management tool for farmers to minimize excretion of N and emission of ammonia whereas its usefulness for government policy is restricted due to factors that affect MUN such as salt intake and diurnal variation in MUN caused by the time and frequency of milking and feeding that are difficult to monitor by the government.

Future Research

For an improved understanding of variation in MUN that is not related to variation in UUN, two areas for future research remain: 1) acquiring a better quantitative insight in factors that affect GFR and RRR and 2) to determine and quantify the causes of high positive N-balances. Both areas are discussed below.

Variation in GFR and RRR

Although some useful empirical equations could be established for dietary effects on GFR and RRR (and for the effect of GFR and RRR on the relationship between MUN and UUN), still much variation between studies remain (Figures 2 – 5). There is little quantitative information on factors that may cause such differences in GFR and RRR, and further research is needed to identify these factors in the lactating cow as the target animal. Furthermore, no information on GFR and RRR is available at CP values lower than 11.5% or higher than 15.5% in lactating dairy cattle. More information is required with respect to main effects and interaction effects of factors such as body weight (urea distribution volume, with perhaps separate compartments to be distinguished), water consumption, water restriction (for certain hours of the day), ambient temperature and day length (Tsuda et al., 1995), salt intake, protein intake, diurnal patterns in feed intake, and other factors that may affect renal function and urea excretion in urine.

N-balance

In general, positive N-balances are observed as demonstrated in a meta-analysis by Spanghero and Kowalski (1997) and also observed in the studies presented in this thesis (Chapters 3, 4, and 5) that cannot be explained by changes in BW. Furthermore, the size of these N-balances appears positively related to intake of digestible N. It is possible that part of this N remains unaccounted for because it is excreted in forms that are not measured by Kjeldahl analysis such as gaseous N_2 (Costa et al., 1968) and nitrate in urine and feces (Kurzer and Calloway, 1981). At present, it is not clear in what form this unaccounted N is present but considering the fact that this quantity can be substantial (e.g. as large as 14% of N-intake in Chapter 5) it

requires further investigation. The analysis discussed in this chapter in the section ‘Effects of positive N-balances on establishing MUN – UUN relationships’ which investigated the effect of a positive N-balance using two different assumptions, suggests that the positive N-balance has to be explained by a fraction of urea that is excreted in urine but for a reason unknown (urine was acidified at sampling moments) remains undetected in urine collected and sampled.

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Summary

Policy by the Dutch government is directed towards reducing nitrogen (N) excretion by dairy cattle due to its substantial contribution to environmental N pollution by nitrate (NO_3) leakage to ground and surface waters, emission of N as ammonia (NH_3) and as nitrous oxide (N_2O), the latter being a major greenhouse gas. Because of the positive relationship between the concentration of milk urea N (MUN) and urinary N-excretion (UN) or urinary urea N-excretion (UUN), MUN seems to be a good candidate for an indicator to be used in farm management as a tool to minimize UUN and thereby reduce N emissions. However, data from N-balance trials show that a significant proportion of variation (28%) in UN remains unexplained by MUN content (with MUN ranging from 5 to 30 mg urea N/dL milk). Upon using a subsample of this dataset in a limited MUN range of 5 to 15 mg N/dL often observed in practice, prediction of urine N-excretion by MUN is even worse (77% unexplained variation). The aim of this thesis was to increase the applicability of MUN as a predictor of UN or UUN by identifying and quantifying factors that can explain variation in MUN that is unrelated to variation in UN and UUN.

In order to identify the factors that affect the relationship between MUN and UUN a literature study was carried out (Chapter 2). A number of factors were identified that affect the relationship between MUN and UUN including dietary crude protein (CP) content, intake of dietary salt or water, body weight, diurnal variation in plasma urea N concentration (PUN), exchange of urea between blood and milk, and heritability of MUN. Accounting for such factors in the relationship between MUN and UUN might substantially improve the applicability and accuracy of MUN as a predictor of protein utilization efficiency and UUN.

Besides this qualitative review of factors affecting the relationship between MUN and UN or UUN, a quantitative meta-analysis was carried out to investigate the effect of various physiological and dietary factors on the relationship of MUN with UN or UUN (Chapter 3). In this meta-analysis it appeared that the combination of MUN and CP explained more variation in UN or UUN than either MUN or CP alone. With a dataset that contained only observations that were based on quantitative collection of urine, the R^2 value of the relationship between MUN and UN increased from 0.85 to 0.93 when CP was included next to MUN. Likewise, the R^2 value of the relationship between MUN and UUN increased from 0.93 to 0.96 when CP was included in the equation next to MUN. Furthermore, coefficients for MUN changed in the relationships established between UN and UUN. This meta-analysis also showed that in view of precision and accuracy, prediction equations for UN and UUN should be derived from quantitative collected urine instead of being based on zero N balance assumptions.

One of the factors established in the literature review to affect the relationship between MUN and UN or UUN is dietary salt content or drink water intake. In order to quantify this effect of dietary salt on MUN and UUN a repeated Latin square experiment was carried out with 12 dairy cows in which the effect was tested of four dietary levels of sodium chloride (NaCl) on urea levels in blood plasma and milk and on UN and UUN (Chapter 4). A negative

relationship was established between dietary Na intake and MUN (MUN (mg N/dL) = 13.5±0.35 − 0.0068±0.00104 × Na intake (g/d)), whereas UUN remained unaffected and only UN slightly increased with NaCl intake due to an increased non-urea N excretion in urine. The level of NaCl intake should, therefore, be taken into account when MUN is used as an indicator of UUN excretion by dairy cows.

Having established the effect of dietary NaCl on MUN, it remained unclear whether this effect would remain the same with varying CP as the renal mechanism of excretion and reabsorption of urea is affected by both CP and NaCl intake. Therefore, the interaction between the intake of NaCl and protein on MUN and UUN was tested in an experiment with a split plot design using 12 dairy cows, testing two CP levels (11.6 and 15.4 % CP in DM) as the main plot, and two dietary NaCl levels (3.1 and 13.5 g Na/kg DM) as the subplot (Chapter 5). No interaction was found, however, between intake of NaCl and protein on MUN, UUN and renal clearance characteristics. In line with the previous study, the relationship between MUN and UUN was again affected by NaCl intake: UUN = -17.7±7.24 + 10.09±1.016 × MUN + 2.26±0.729 × MUN (corrective term for the high NaCl diet only); $R^2 = 0.85$. Removal of the MUN × NaCl interaction term lowered the coefficient of determination from 0.85 to 0.77. Besides, there was no interaction between protein content and NaCl intake on plasma urea entry rate and urea transfer to the gastro intestinal tract.

The literature study also revealed that the diurnal variation in PUN and MUN can be substantial, as a result of time and frequency of feeding and milking. Insight in the dynamics of urea transport between blood of milk is hence important to predict variation in MUN over time under different feeding and milking regimes. To obtain insight in urea transport between blood and milk two studies were conducted. A pilot study (Chapter 6) demonstrated applicability of the experimental measurement protocol to test the dynamics of urea transfer from milk to blood and of urea transfer within the mammary gland. The protocol comprised analysis of the disappearance of a cistern injected pulse dose of cistern injected [^{15}N^{15}N]urea from milk and the time of appearance in blood after injection. As well, the distribution of cistern injected [^{15}N^{15}N]urea toward alveoli milk was studied. In the follow-up main study, (Chapter 7) the dynamics of disappearance of cistern injected pulse doses of [^{15}N^{15}N]urea from milk was studied at four levels of MUN together with the distribution patterns of cistern injected [^{15}N^{15}N]urea throughout the milk volume in the mammary gland. The fractional urea disappearance rate from milk to plasma was unaffected by urea concentration in blood or milk (although the fractional urea disappearance rate of urea from milk to blood plasma tended (P=0.079) to be positively related to MUN). The fractional disappearance rates of labeled urea from milk varied between 0.456 and 0.576 /h for the pilot study and between 0.425 and 0.666 /h for the main study. In both the pilot and the main study the cistern injected labeled [^{15}N^{15}N]urea was distributed rapidly (within 20 min) throughout the entire distribution volume of milk (cistern, ducts, and alveoli). A comparison to literature results indicates that

the measured value of the fractional disappearance rate of urea from milk to plasma can be used to quantify the response of MUN to diurnal variation in PUN.

In the general discussion the principal role is discussed of the function of the kidneys in determining the relationship between MUN and UUN. Factors that affect the glomerular filtration rate (GFR) and the renal urea recycling ratio of urea (RRR) also affect the relationship between MUN and UUN. Based on a meta-analysis including data from sheep, beef, and dairy cattle it is concluded that intake of water and salt, CP content, and body weight affected GFR whereas only CP clearly affected RRR. The effects of body weight, CP, and intake of water and salt, mediated through RRR and GFR, on MUN and the UUN-MUN relationships were modeled. The calculated effect of CP and urine production on MUN and UUN resulted in UUN-MUN relationships similar to those reported in literature. Furthermore, the effect of body weight, mediated trough GFR, on UN-MUN relationships was quantified and compared to results from an in vivo study with cows. Calculated effect of BW on the UN-MUN relationship was similar to the results reported in the in vivo study. Finally, because of some strongly positive N balances obtained in one of the trials as part of this thesis, the consequences of the presence of a positive N-balance on the UUN-MUN relationship was established via two approaches. First, it was assumed that the positive N-balance was due to urea excreted in urine but for unknown reasons not measured. Second, it was assumed that the positive N-balance was due to non-urea losses. A comparison between the simulated UUN-MUN relationships and the UUN-MUN relationship observed in the meta-analysis of in vivo data as part of this thesis (Chapter 3) suggests that the positive N-balance is due to urea excreted in urine and that this N was hence not measured with analysis for reasons that remain unknown. From the discussion it is concluded that modeling efforts that aim to quantify the effect of nutrition, management, and cow characteristics on MUN and on the relationship between MUN and UUN, should account for renal mechanisms involved with N excretion, including GFR and RRR. Future research should focus on the relationship of physiological, and nutritional factors with GFR and RRR, and on the interactions between these factors. Another important topic for future research is to resolve the precise background of (high) positive N-balances that becomes apparent in feeding trials.

The overall conclusion of this thesis is that the applicability of MUN as a predictor of UUN or UN can be increased by taking into account the factors that are involved with the regulation of renal urea N excretion and the diurnal dynamics of urea in blood.

Samenvatting

Het Nederlandse overheidsbeleid is gericht op het reduceren van stikstof (N) uitstoot door melkvee vanwege het substantiële aandeel van deze diergroep aan milieuvervuiling van N in de vorm van nitraat (NO_3) in grond- en oppervlaktewater, emissie van N in de vorm van ammoniak (NH_3) en emissie van het belangrijke broeikasgas lachgas (N_2O). Vanwege de positieve relatie tussen de concentratie van melkureum-N (MUN) en uitscheiding van urine-N (UN) en urine-ureum-N (UUN) zou MUN op bedrijfsniveau gebruikt kunnen worden als een instrument om UUN, en daarmee uitstoot van N in het milieu, te minimaliseren. Resultaten van N-balans proeven waarbij MUN varieerde van 5 tot 30 mg N/dL laten echter zien dat een aanzienlijk deel van de UN variatie (28%) niet verklaard kan worden door MUN. Het aandeel verklaarde variatie in UN door MUN was echter nog een stuk lager (77% onverklaarde variatie) wanneer een beperkte dataset gebruikt werd met alleen waarnemingen van MUN met een range tussen 5 en 15 mg N/dL, een range die vaak voorkomt in de praktijk. Het doel van dit promotietraject was het vergroten van de bruikbaarheid van MUN als een indicator voor UN en UUN door middel van het identificeren en kwantificeren van factoren die dat deel van de variatie in MUN kunnen verklaren wat niet is gerelateerd aan UN of UUN.

Om de factoren te identificeren die de relatie tussen MUN en UUN of UN beïnvloeden werd een literatuurstudie uitgevoerd (Hoofdstuk 2). Uit dit literatuur onderzoek kwamen een aantal factoren naar voren die de relatie tussen MUN en UN of UUN beïnvloeden zoals eiwitgehalte in het voer (RE; % in droge stof), opname van zout of drinkwater, lichaamsgewicht, binnen dag variatie in plasma ureum-N gehalte (PUN), uitwisseling van ureum tussen bloed en melk en genetische variatie. Door rekening the houden met deze factoren bij het bepalen van de relatie tussen MUN en UUN kan de nauwkeurigheid en toepasbaarheid van MUN als schatter van UUN verbeteren.

Naast deze kwalitatieve literatuurstudie over factoren die de relatie tussen MUN en UN of UUN beïnvloeden werd een kwantitatieve meta-analyse uitgevoerd naar de effecten van verschillende fysiologie- en rantsoenfactoren op de relatie tussen MUN en UN en tussen MUN en UUN (Hoofdstuk 3). In deze meta-analyse werd duidelijk dat de combinatie van de fysiologische factor MUN en de rantsoenfactor RE aanzienlijk meer variatie in UN en UUN kan verklaren dan elk van beide factoren afzonderlijk. Zo steeg, gebaseerd op de dataset die alleen waarnemingen bevatte van studies waar urine kwantitatief verzameld werd, de R^2 van de relatie tussen MUN en UN van 0.85 naar 0.93 wanneer naast MUN ook RE als een variabele werd meegenomen in het model. Eveneens steeg de R^2 van de relatie tussen MUN en UUN van 0.93 tot 0.96 wanneer naast MUN ook RE als variabele werd meegenomen in het model. Ook veranderde de regressie-coëfficiënt van MUN voor de relatie tussen MUN en UUN. Uit resultaten van deze meta-analyse bleek ook dat voor het nauwkeurig bepalen van de relatie tussen MUN en UN de analyse van UN op basis van kwantitatieve verzameling van urine plaats moet vinden.

Uit de literatuurstudie bleek dat de opname van zout of drinkwater één van de factoren is die de relatie tussen MUN en UUN of UN beïnvloeden. Om het effect van zoutopname op

MUN en UUN te kwantificeren werd een Latijns vierkant proef opgezet met 12 melkkoeien waarbij het effect van vier gehalten aan natriumchloride (NaCl) in het rantsoen werd onderzocht op UN, UUN en MUN (Hoofdstuk 4). Er werd een negatieve relatie tussen Na opname en MUN gevonden: MUN (mg N/dL) = 13.5±0.35 − 0.0068±0.00104 × Na opname (g/dag), terwijl UUN niet beïnvloed werd door Na opname en UN lichtjes toenam met NaCl opname veroorzaakt door een toename van niet ureum-N excretie in urine.

Omdat het mechanisme in de nieren, wat ureumuitscheiding in urine en ureumreabsorptie terug naar bloed betreft, beïnvloed wordt door de consumptie van zowel zout als eiwit kwam de vraag naar boven of het effect van NaCl op MUN hetzelfde is bij hoge en lage RE. Om de interactie tussen opname van zout en eiwit op MUN en UUN te testen werd een split plot experiment opgezet met 12 melkkoeien waarbij als hoofdplot twee RE niveaus (11.6 en 15.4 %) en als subplot twee NaCl niveaus (3.1 en 13.5 g Na/kg droge stof) werden getest (Hoofdstuk 5). Er werd geen interactie tussen RE en NaCl op MUN, UUN en nier processen gevonden. Net als in de voorgaande studie werd de relatie tussen MUN en UUN beïnvloed door NaCl opname: UUN (g N/dag) = -17.7±7.24 + 10.09±1.016 × MUN (mg N/dL) + 2.26±0.729 × MUN (mg N/dL) (voor hoog NaCl); R^2 = 0.85. De verwijdering van de MUN × NaCl interactie uit het model verlaagde de determinatiecoëfficiënt van 0.85 naar 0.77.

Uit de literatuurstudie bleek dat er op dag basis grote schommelingen in MUN en plasma ureum-N concentratie (PUN; mg N/dL) kunnen plaatsvinden en dat deze schommelingen afhankelijk zijn van factoren zoals het moment en de frequentie van voeren en melken. Inzicht in de dynamiek van ureumtransport tussen bloed en melk is van belang om schommelingen in MUN bij verschillende voer- en melkregimes op dag basis te kunnen modeleren. Om inzicht te krijgen in ureumtransport tussen bloed en melk werden twee proeven uitgevoerd. In een pilotproef (Hoofdstuk 6) werd een experimentele werkwijze getest voor het bepalen van ureumfluxen van melk naar bloed en van ureumverplaatsing in het uier zelf. In deze pilotproef werd de verdwijningssnelheid geanalyseerd van geïnjecteerde puls bolussen van [^{15}N^{15}N]ureum uit melk en het tijdstip van verschijnen van ^{15}N-ureum in bloedplasma na injectie in het uier. Daarnaast werd ook de verplaatsing van in de melkboezem geïnjecteerd [^{15}N^{15}N]ureum richting alveolimelk gemeten. In de hoofdstudie (Hoofdstuk 7), werd d.m.v. een Latijns vierkant proefopzet met vier koeien de dynamiek van verdwijning van in de melkboezem geïnjecteerd [^{15}N^{15}N]ureum gemeten bij vier verschillende MUN niveaus en werd tevens de verplaatsing van in de melkboezem geïnjecteerd [^{15}N^{15}N]ureum richting alveolimelk gemeten. Uit de hoofdstudie bleek dat de fractionele verdwijningssnelheid van melk naar plasma niet afhankelijk was van MUN (alhoewel er wel een tendens (P=0.079) was voor een positieve relatie tussen MUN en de fractionele verdwijningssnelheid van ureum uit melk). De fractionele verdwijningssnelheid uit melk van gelabeld ureum varieerde tussen 0.456 en 0.576 /uur voor de pilotproef en tussen 0.425 en 0.666 /uur voor de hoofdstudie. In zowel de pilotproef als in de hoofdproef bleek in melkboezem geïnjecteerd [^{15}N^{15}N]ureum zich snel (binnen 20 min) te verspreiden in de melk aanwezig in de melkboezem,

melkkanaaltjes en melkklieren. Uit een vergelijking met resultaten uit de literatuur lijkt het er verder op dat de gemeten fractionele verdwijningssnelheden van ureum uit melk naar plasma gebruikt kunnen worden voor het kwantificeren van MUN als een gevolg van binnen dag variatie in PUN.

In de algemene discussie wordt de centrale rol van de nieren in het bepalen van de relatie tussen MUN en UUN bediscussieerd. Factoren die de glomerulaire filtratiesnelheid (GFR) en de fractie van ureum dat uit de voorurine weer gerecycled wordt naar bloed (RRR) beïnvloeden hebben ook een effect op de relatie tussen MUN en UUN. Op basis van een meta-analyse met observaties van schapen, vleesvee en melkvee bleek dat de factoren RE, lichaamsgewicht en de opname van drinkwater en zout GFR beïnvloeden terwijl de factor RE als enige factor een duidelijk effect had op RRR. De effecten van RRR en GFR, beïnvloed door lichaamsgewicht, RE en opname van drinkwater en zout op MUN en de UUN-MUN relatie werden gemodelleerd. Uit modelsimulaties bleek dat het effect van RE en urineproductie op MUN en UUN resulteerde in UUN-MUN relaties die vergelijkbaar zijn met UUN-MUN relaties die in de praktijk worden waargenomen. Verder bleek dat het effect van lichaamsgewicht op de UUN-MUN relatie vergelijkbaar was met resultaten van een in vivo studie. Als laatste werd de aanwezigheid van een positieve N-balans gemodelleerd via twee benaderingen. Bij de eerste benadering werd aangenomen dat de positieve N-balans werd veroorzaakt door ureum dat was uitgescheiden in de urine maar niet is gemeten. Bij de tweede benadering werd aangenomen dat de positieve N-balans werd veroorzaakt door N-verliezen anders dan ureum-N in urine. Een vergelijking tussen de gesimuleerde UUN-MUN relaties en de UUN-MUN relatie gevonden in de meta-analyse in hoofdstuk 3 die is gebaseerd op in vivo data suggereert dat een positieve N-balans het beste verklaard kan worden door ureumuitscheiding in urine wat om onbekende redenen niet wordt gemeten. Op basis van de informatie in het Algemene Discussie hoofdstuk wordt geconcludeerd dat bij pogingen om effecten van voeding, management en koe eigenschappen op de relatie tussen MUN en UUN te kwantificeren rekening moet worden gehouden met nierprocessen die betrekking hebben op ureumexcretie zoals de GFR en RRR. Vervolgonderzoek zou gericht moeten zijn op de effecten van nutritionele en fysiologische factoren die GFR en RRR beïnvloeden. Een ander belangrijk onderwerp voor vervolgonderzoek is het ophelderen van de oorzaak van de in het algemeen gevonden positieve N-balansen in N-balans proeven.

De algehele conclusie van dit proefschrift is dat de toepasbaarheid van MUN als een schatter van UUN en UN vergroot kan worden indien rekening wordt gehouden met
1) factoren die een rol spelen in het bepalen van binnen dag variatie in PUN en
2) het regulatiemechanisme van de nieren om ureum uit te scheiden in ureum.

Dankwoord

Ruim vijf jaar terug kreeg ik een telefoontje van Wouter Hendriks met de mededeling dat er een aantal AIO vacatures bij Diervoeding beschikbaar waren en de vraag of ik interesse had voor één van deze vacatures. Die interesse had ik met dit proefschrift als gevolg en daarnaast een leerzame tijd waarin ik mij verder kon bekwamen als onderzoeker. Dank Wouter voor dat telefoontje en voor je stimulerende houding gedurende het hele promotietraject. Dit voorwoord wil ik verder gebruiken om een groot aantal personen te bedanken.

Boven alles gaat mijn dank uit naar mijn God en Vader, de Schepper van hemel en aarde voor Zijn goedheid en zorg zodat ik de gezondheid en mogelijkheid had om dit proefschrift te maken.

Verder wil ik vooral Andre Bannink en Jan Dijkstra bedanken voor hun begeleiding, de prettige omgang met hen, en de waardevolle discussies die we mochten hebben. Andre, bedankt voor je toegankelijkheid en de vele discussies die we gevoerd hebben met betrekking tot dit onderzoek maar ook voor de gesprekken die los hiervan stonden. Jan, bedankt voor je begeleiding en gedetailleerde commentaar op conceptrapporten.

Ook wil ik de mensen van het lab bedanken die meer tijd aan de planning, begeleiding en analyse van monsters hebben besteed dan van te voren gepland. Michel, Leon, Saskia, Jane-Martine, Suang-Hong, Erika en Adriaan, jullie worden hartelijk bedankt.

Verder wil ik al mijn vroegere AIO collega's; Daniel, Judith, Esther, Sonja, Harma, Lotte, Sabrina, Sandra, Kasper, Omid, Shafquat, Nazir, Mubarak, Mascha, Antoon, Christiaan, Sanne, Tetske, Attje-Rieke, Bayissa, Myrthe, Genet, Sergio, Geronda, Henk, Hsuan en Yvonne bedanken voor de prettige tijd die we met elkaar mochten hebben. Ook mijn Lelystad collega's wil ik bedanken, in het bijzonder Gert maar ook Arie, Gerrit, Roselinde, Ad, Ronald en Martin voor hun collegialiteit en hulp.

Verder wil ik Betty en Yvonne van harte bedanken voor hun hulp bij alle regeldingetjes en administratieve zaken die komen kijken bij een promotietraject, wil ik Sven, Tamme, Dirk, Gerrit Jan en Henk bedanken voor hun hulp en praktische adviezen met betrekking tot monstername en het uitvoeren van infusieproeven, wil ik Gerrit bedanken voor de statistische begeleiding, Joost voor zijn advies met betrekking tot het gebruik van stabiele isotopen en Jaap van Zwieten voor zijn hulp bij het samenstellen van de proefvoeders. Ook wil ik Mirian bedanken voor het nemen van foto's van plassende koeien en voor het ontwerpen van de omslag. Waarschijnlijk zijn er een aantal mensen die ik vergeten ben te noemen in dit voorvoord maar die toch op één of andere manier hebben bijgedragen aan dit proefschrift. Bij deze ook jullie bedankt!

Als laatste wil ik Mieke bedanken voor het feit dat ze veel van het achterstallig onderhoud aan ons huis voor lief heeft genomen tijdens dit promotietraject.

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Curriculum Vitae

Jan Wouter Spek (Wouter) was born on June 25th, 1980 in Dronten. In 1997 he graduated from the secondary school "Willem de Zwijger" in Schoonhoven . In the same year of graduation he started his BSc Tropical Agriculture at the professional University "Larenstein" in Deventer with as specialization Small Scale Animal Husbandry. After graduation at Larenstein in 2001 he worked from 2002 to 2005 as a volunteer in Moldova and was involved in an agricultural project run by a local Christian association. In 2005 he started his MSc Animal Nutrition at Wageningen University. After obtaining his MSc degree in 2007 he worked as a nutritionist for the feeding company O. Bouman in Andel and as researcher for the feeding company De Heus after the company O. Bouman was taken over by De Heus. In 2008 he started his PhD at the Animal Nutrition Group of Wageningen University and at the Department Animal Nutrition from Wageningen UR Livestock Research in Lelystad. Since 2013 he is working for the Dutch CVB as a nutritionist.

Jan Wouter Spek (Wouter) is op 25 juni 1980 in Dronten geboren. In 1997 behaalde hij zijn HAVO diploma aan de "Willem de Zwijger" in Schoonhoven. In datzelfde jaar begon hij zijn bachelor opleiding Tropische landbouw met als specialisatie Kleinschalige Veehouderij aan de hogeschool Larenstein in Deventer. Na zijn afstuderen in 2001 was hij van 2002 tot 2005 als vrijwilliger actief in Moldavië. Daar was hij werkzaam bij een kleinschalig landbouwproject van een lokale Christelijke organisatie. In 2005 begon hij zijn master opleiding Diervoeding aan de Wageningen Universiteit. Na het behalen van zijn masterdiploma in 2007 was hij als nutritionist werkzaam bij het toenmalig mengvoerbedrijf O. Bouman in Andel en als onderzoeker bij mengvoerbedrijf De Heus na overname van O. Bouman door De Heus. In 2008 begon hij aan een promotietraject bij de vakgroep Diervoeding van de Wageningen Universiteit en bij de afdeling Diervoeding van de Wageningen UR Livestock Research in Lelystad. Vanaf 2013 is hij als nutritionist werkzaam bij het Productschap Diervoeder voor de CVB-activiteit.

Training and Supervision Plan

		Graduate School WIAS
Name:	J.W. Spek	
Project:	Variation of milk urea in dairy cattle	
Group:	Animal Nutrition	
Daily supervisors:	Dr A. Bannink and Dr J. Dijkstra	
Supervisor:	Prof. Dr W.H. Hendriks	

	Year	Credits*
The Basic Package		
WIAS Introduction Course	2009	1.5
Course on Philosophy of Science and/or Ethics	2009	1.5
International Conferences		
Annual ADSA Meeting, New Orleans, USA	2011	1.5
Eight International Symposium on the Nutrition of Herbivores, Wales, England	2011	1.5
Seminars and Workshops		
34th ANR Forum in Melle, Belgium	2009	0.3
35th ANR Forum in Lelystad, the Netherlands	2010	0.3
36th ANR Forum in Leuven, Belgium	2011	0.3
WIAS Science Day in Wageningen, the Netherlands	2011	0.3
37th ANR Forum in Wageningen, the Netherlands	2012	0.3
WIAS Science Day in Wageningen, the Netherlands	2012	0.3
Nutritional Management in Early Lactation, Wageningen, The Netherlands	2012	0.3
Presentations		
Oral presentation at WIAS Science day 2011	2011	1.0
Poster presentation at ANR Forum in Leuven, Belgium	2011	1.0
Oral presentation at Annual ADSA Meeting in New Orleans, USA	2011	1.0
Poster presentation at ISNH8 in Wales, England	2011	1.0
Oral presentation at ANR Forum in Wageningen, the Netherlands	2012	1.0
In-Depth Studies		
Analytical Work and Possibilities within Animal Nutrition Sciences	2009	1.0
Advanced Statistics Course: Design of Animal Experiments	2009	1.0
Reaction Kinetics in Food Science	2010	1.5
Statistics for the Life Sciences	2010	2.0
Mixed Linear Models	2011	0.6
Epigenesis and Epigenetics	2011	0.8
Statutory Courses		
Use of Laboratory Animals	2009	3
Professional Skills Support Courses		
Techniques for Scientific Writing	2010	1.2
Project and Time Management	2012	1.5
Presentation Skills	2012	1.0
Supervising Practicals		
BSc Practical Rumen Physiology	2010 - 2012	1.5
MSc Practical Feed Technology	2010	0.5
Supervising Thesis		
Supervising MSc Major Thesis (5 students)	2010 - 2012	9.5
Supervising BSc Thesis (8 students)	2010 - 2011	5.0
Total		**43**

*One ECTS credit equals a study load of approximately 28 hours

Colophon

The research described in this thesis was financially supported by the Dutch Dairy Board (PZ), The Dutch Product Board Animal Feed (PDV), The Dutch Ministry of Economic Affairs, Agriculture and Innovation (EL&I), and Wageningen UR Livestock Research. Financial support from Wageningen University and Wageningen UR Livestock Research for printing this thesis is gratefully acknowledged.

Cover: Mirian Hendriks
Print: GVO drukkers & vormgevers B.V. | Ponsen & Looijen, Ede, the Netherlands