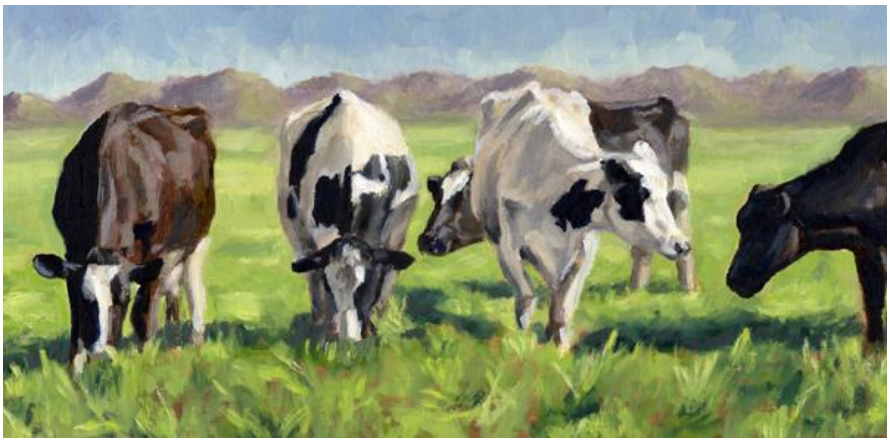
Effect of Dietary Salt Intake on Milk Urea Level in Dairy Holsteins



Shaojie Zhuang
Applied Animal Science
Van Hall Larenstein
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Abstract

Ammonia emission is a world wide environmental problem. In the Netherlands agriculture is a major source of ammonia emission, and more than 20% is originated from the cattle sector, majorly by urinal urea. Reducing urea-N excretion is therefore very important for environment protection. The present study investigated the effect of salt (NaCl) intake on urea-N excretion by using milk urea level as an indicator. Sixteen multi-parity Dutch dairy Holsteins (daily milk production 27.74 ± 3.14 kg and body weight 654.9 ± 43.1 kg) were used in a 4x4 crossover design with treatment varying in four levels of additive dietary salt (0, 0.333, 0.667 and 0.999 kg/cow/day). Salt level was positively correlated with daily water intake (R^2 = 0.527) and urine production (R^2 = 0.628), whereas daily feed intake was not affected. Milk urea level was negatively correlated with dietary salt level; the difference of milk urea concentration between 0 and 0.999 kg/cow/day additive salt was approximately 5.333 mg/dl. Stall type also had an effect on milk urea in this study.

Key words: ammonia, N excretion, milk urea, dietary salt

Annex

Introduction

Ammonia emission is currently one of the most important worldwide environmental issues. The major problems focus on acidity of precipitation and soil eutrophication, which may further disturb the terrestrial and aquatic ecosystem. Because of the international impact of ammonia emission, the issue was addressed in the Gothenburg Protocol in 1999, which aimed at a long term protection of health and ecosystems by bringing down emissions below critical loads and levels (CIAM, 2007). As one of the countries with highest ammonia emission levels in Europe, the Netherlands plays an important role in the achievement of the Gothenburg Protocol. Agriculture is a primary source of ammonia emission in the Netherlands. More than half of the emission within agriculture comes from animal housing, and more than 20% is contributed by cattle sector (CBS, 2010). Other major sources are manure and fertilizer application in the pastures (Bussink and Oenema, 1998). Regarding ammonia emission produced by dairy farms, the Dutch Farmers Union Land en Tuinbouw Organisatie Nederland (LTO) has made an agreement to reduce dietary N content by 2010 in order to reduce urea excretion (Melse et al., 2009).

Urinal urea from dairy cattle is a primary source of ammonia emission (Bussink and Oenema, 1998). Several studies have confirmed the relationship between milk urea level and urinal urea level (Jonker et al., 1998; Kauffman and St-Pierre, 2001). Comparing to faeces and urine, it is easier to collect milk samples and thereafter to measure milk urea. With the development of automated milking systems and sensor devices, the effort for milk urea measurement will be remarkably reduced. Therefore, milk urea has a great potential to become a useful tool for ammonia emission monitoring and control.

The main sources of milk urea are dietary protein and metabolite of body tissue, so in current farming system milk urea level is normally used by nutritionists and veterinarians as an indicator of dietary protein/energy balance, and sometimes also used for monitoring rumen microbial function. Moreover, early studies also suggested that milk urea level was correlated with breed, season, lactation stage, parity, health statue, water intake, etc. In order to have a better understanding and interpretation towards milk urea level, it is important to understand it is influenced by this factors.

Dietary factors, as protein/energy balance and water intake, are more controllable by farm management, and therefore the understanding of them will be more practically meaningful. Protein/energy balance has been well studied and the relevant knowledge is already applied in feeding management. milk urea could reflect urinal and fecal N excretion in case of excessive crude protein intake, and therefore milk urea level could be a useful tool for measuring environmental pollution caused by N loss (Jonker and Kohn, 2001). In contrast the understanding towards water intake is still insufficient. Early studies found that restricted water intake could increase urea level in the milk (Utley et al., 1970; Burgos et al., 2007), while it was unknown if increased water intake would have a reverse effect. Moreover, it was also uncertain if water intake would influence the correlation between milk urea level and urinal urea level. In practice it is very difficult to force animals to drink more water, but higher salt intake can stimulate drinking behavior. The aim of the present study was to investigate the effect of salt intake on milk urea level and its relationship with N-excretion in dairy Holsteins, and four sub-questions were studied specifically to reach the aim:

1. What is the importance of reducing urea-N excretion in terms of environment and climate change?
2. What is the physiological basis of urea production in dairy cattle?
3. What is the effect of salt intake on milk urea?
4. Does the correlation between milk urea and urinal urea remain high under the influence of dietary salt?

Literature review

Ammonia emission

Ammonia is a nitrogen-hydrogen based compound, with the formula NH_3. It is highly miscible with water to form ammonium hydroxide, with the formula $NH_3 \cdot H_2O$. The dilution ratio with water is 1:700 under standard atmospheric pressure (100 kPa) and normal temperature (20 ℃). Ammonium hydroxide is moderately basic. While being heated or in light, it can be degraded and release ammonia. Ammonia is an important resource of food and fertilizer to the living organisms.

With the development of agriculture, ammonia emission has become a worldwide environmental issue. Emitted ammonia can cause acidity of precipitation, cloud water and atmosphere aerosol. Deposition of ammonia also plays an important role in soil eutrophication due to excessive N, and further affects the terrestrial and aquatic ecosystem (Aneja et al., 2001). Estimation in 2000 showed that 93% and 84% of the ecosystem in the Netherlands was unprotected for eutrophication and acidification respectively (CIAM, 2007), and thus for maintaining the Dutch ecosystem it is very important to restrict local ammonia emission. Furthermore, atmospheric ammonia can be transported with air masses up to thousands of kilometers, and environmental damage may still occur, so monitoring and control of ammonia emission is also a universal issue.

In an European Monitoring and Evaluation Program (EMEP) Assessment Report, (Lövblad et al., 2007) studied the ammonia emission from 1980 to 2000. The report pointed out that most ammonia emission was from large countries with extensive agriculture, and the Netherlands was one of the major contributors, for whatever atmospheric ammonia, precipitation and deposition. Looking at the European as a whole, the total ammonia emission reduced by 20% from 1980 to 2000, and the reduction in the Netherlands was 13%. The major causes were improved fertilizer application measures, more efficient N utilization from feed and reduced cattle number (Lövblad et al., 2007). In 1980s a voluntary management tool (an input-output formulation tool) for dairy farmers was introduced in the Netherlands in order to reduce N surplus on farm level (Hanegraaf and den Boer, 2003), and it played an important role in monitoring and reducing ammonia emission. Total cattle number and farm number dropped rapidly since 1980s. From 1984 to 2004 cattle number reduced from 5.5 million to 3.8 million, and the decreasing was mostly in dairy cattle (including replacement young cattle); farm number reduced by half from 80 thousand to 40 thousand (CBS, 2005). Although meanwhile both pig and poultry increased in numbers (CBS, 2003; CBS, 2005), the reduction in cattle sector still overcame other sectors and lead to declined ammonia emission.

In 1999, a Gothenburg Protocol, as a part of process of the Convention on Long-range Transboundary Air Pollution (LRTAP), was signed by 23 European countries, including the Netherlands. In this protocol, emission limits were set for ammonia and other pollutive substances. The first review of the Gothenburg protocol assessed the achievement in 2005 by

using a Regional Air Pollution Information and Simulation (RAINS) model to monitor current achievements and to predict future emissions in 2010 and 2020 (CIAM, 2007). The results showed that ammonia emission in the Netherlands would be 4% ($1.23x10^8$ kg/year vs. $1.28x10^8$ kg/year) lower than the limit by 2010, but would be 8% higher ($1.38x10^8$ kg/year vs. $1.28x10^8$ kg/year) than the limit by 2020. The results suggested that although the emission in the Netherlands was temporally low, the trend had turned up-going, and suppression was therefore necessary.

Vries et al. (2002) studied the impact of animal production on ammonia emission by using field data in the Netherlands. They concluded that 2/3 of the ammonia emission was from livestock housing, and the rest were from manure and fertilizer application. More recent statistical data by CBS (2010) showed that indoor housed farm animals contributed to more than half of the ammonia emission produced by agriculture in the Netherlands, and more than 20% of the total emission was from cattle housing (Figure 1). Within the Dutch cattle sector, only 5% of the animals were kept in low-emission housing instead of traditional housing.

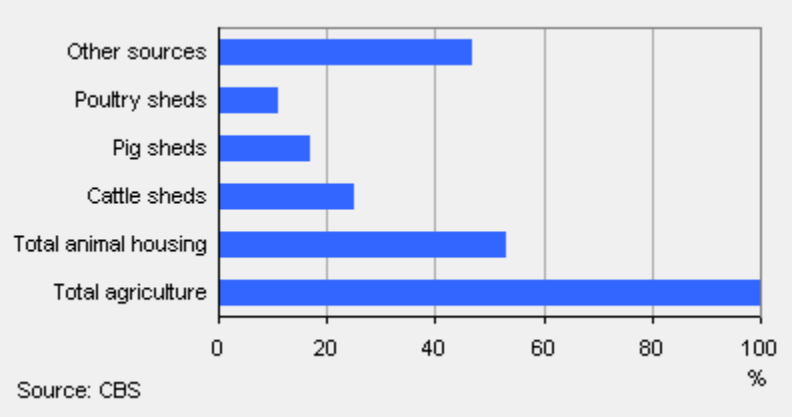

Figure 1 Agricultural ammonia emission in the Netherlands. Source CBS, 2010

Bussink and Oenema (1998) reviewed early studies in terms of several potential N loss (in form of NH_3) sources in dairy farming. In their review the measured ranges of N loss from housing, slurry application and fertilizer application were respectively 0-70%, 1-100% and 6-42%. Their review did not explain what caused the range, but several guesses could be made. For housing, the low N loss could be resulted by low-emission housing system, and in contrast high emission was due to conventional system. Nevertheless, it was doubtful if low-emission housing system could completely prevent N loss. For slurry and fertilizer application, the N loss could related to

soil type, crop/grass type, weather, application methods, etc., so it would be more difficult to interpret the variance. Urine and manure were the primary source of N loss in housing. Up to 50-90% N in urine was urea-N, and urea had the highest volatilization potential comparing to the other N source. 4-41% N from cattle urine may volatize, with an extreme of 66%. N volatilization from feces, on the other hand, was rather low because a limited amount of N would be converted into urea and other volatile compounds; only 1%-13% N from feces may volatize.

Nitrogen metabolism in Ruminants

A simple N path through ruminates was decrypted by Round and Herd (unknown publish year). The most important resource of N in diet is protein, while feed can also contain other non-protein N (NPN). Protein includes rumen degradable protein (RDP), bypass protein and undigested protein. RDP and NPN are transformed into ammonia by microbes in the rumen, and there the ammonia can either be further converted into microbial protein which afterwards enters the small intestine or directly enters the blood stream through the rumen wall. In the blood vessel the ammonia is then transported to the liver and partly converted into urea. Some of the urea becomes urinal urea via the kidney, and the rest of the urea returns to the rumen as NPN either directly or via saliva. The returned urea can again be converted into ammonia. In the small intestine the bypass protein and the microbial protein are converted into amino acids except for the undigested protein. The undigested protein will be finally excreted in feces. The amino acids enter the blood stream through the wall of the small intestine and then will be transported to the liver. There the amino acids are either used for tissue metabolism or converted into ammonia. Through the entire digestion process, N can be recycled in three different tracks:

1) ammonia (rumen) → ammonia (liver) → urea (liver) → ammonia in rumen;
2) ammonia (rumen) → ammonia (liver) → urea (liver) → urea (saliva) → ammonia (rumen);
3) ammonia (rumen) → amino acid (small intestine) → ammonia (liver) → ammonia (rumen).

Based on the trace of N metabolism, two potential strategies could be applied to reduce N excretion: 1) reducing N intake, and 2) enhancing the recycling of N in digestive tract.

In terms of urea production Lapierre and Lobley (2001) suggested the most important factor was the nature of metabolites present in the liver, which was determined by the substances and

N balance in the diet. Moreover, growth and reproductive status also significantly influenced urea-N formation and excretion. Nevertheless, from their point of view the absolute N intake was a rather irrelevant factor regarding urea production. To this point an opposite conclusion was given by Jonker and Kohn (2001), and they found overfeeding of dietary N would finally result in an increased milk and urinal urea excretion.

Milk Urea, Urinal Urea and correlated factors

Early studies showed that milk urea level in dairy cattle was highly correlated with plasma urea level (Thornton and Wilson, 1972; Broderick and Clayton, 1997), $R^2 > 0.91$. The correlation between plasma urea and urinal urea was also strong (Thornton and Wilson, 1972), $R^2 = 0.97$. The R^2 between milk urea and urinal urea was 0.85 and 0.842 respectively reported by Broderick and Clayton (1997) and Zhai et al. (2005). The high correlation can be well explained by physiological urea circulation. Blood vessels are the primary urea flow channel. During milk synthesis, urea is carried with the blood stream to the mammary gland and enters the milk, in proportion to the concentration of blood urea. It is similar for urinal urea that urea is carried with the blood stream to the kidney and then is excreted in the urine.

Milk urea is correlated to various factors. It was found that evening milk urea level was significantly higher than in the morning (Broderick and Clayton, 1997; Godden et al., 2001). A milk urea peak was showed in summer time (Godden et al., 2001; Arunvipas et al., 2003; Johnson and Young, 2003; Rajala-Schultz and Saville, 2003; Hojman et al., 2005). For both time and seasons, temperature difference was a common factor. Muroya et al. (1997) observed that milk urea was higher under a high temperature and high humidity, and this may explain the milk urea difference in time and seasons. However, the physiological basis regarding the temperature effect was not explained. In addition to temperature, the seasonal effect might be partly explained by pasture grazing because of the higher protein/energy content in fresh grass.

Milk urea level is significantly higher when daily water intake is reduced (Utley et al., 1970; Burgos et al., 2007). This finding may have two possible explanations. One is that certain essential functions of water in N metabolism and feed is affected. Less feed intake, namely less N intake, associates with water restriction, and it could lead to both a negative-N balance and negative energy balance. In this case, tissue protein breakdown would be the most possible

source of milk urea. The other explanation is that milk yield is reduced due to less water intake, and consequently the concentration of urea per volume unit is higher. In spite of increased milk concentration, nevertheless, the quantity of excreted urea-N could still be lower when water intake is restricted. However, the quantity of excreted urea-N was not estimated in either study.

Milk urea level has a quadratic correlation with lactation stage, where it reaches a peak at 2-3 months of lactation (Godden et al., 2001; Arunvipas et al., 2003). This may be related to milk yield because yield peak also peaks around 2^{nd} and 3^{rd} month in lactation. A positive correlation between milk urea and yield was shown (Arunvipas et al., 2003; Johnson and Young, 2003; Hojman et al., 2005). Godden et al. (2001) also reported that the relationship between milk urea and yield was also positive but not linear, and using energy-corrected milk could improve the model fit. This correlation may associate with the negative energy balance in early lactation. With a negative balance, body tissue is usually broken down for maintenance and lactation, and consequently additional urea, as metabolite of tissue protein, is secreted in the milk.

First lactation cows tended to have a lower milk urea (difference = 1.77 mg/dl) than multi-parity cows (Godden et al., 2001). The reason could be that heifers were still in growth, and the efficiency of amino acids utilization is therefore higher. Similar results were presented by Arunvipas et al. (2003), but the difference between single and multi-parity cows was numerically small in their study (0.25~0.32 mg/dl).

Statistic models were developed in several studies for milk urea prediction. Broderick and Clayton (1997) developed a linear regression model which included blood urea level, parity, body weight, milk yield, 3.5% fat corrected yield, fat yield, dietary crude protein, excess N intake, dry matter intake, NE_L (required net energy corrected for lactation) intake and lactation day as variables for predicting milk urea, and the model gave $R^2 = 0.875$. The study of Arunvipas et al. (2003) investigated the relationship between milk urea and non-notional factor with a linear regression model, which included days in milk, milk yield, milk fat%, milk protein%, parity, month of the year and cow/herd as fixed variables for predicting milk urea level, and only 13% of variation was explained by the model. The two models above suggested that milk urea was under a major influence of nutritional factors rather than non-nutritional ones.

Material and Methods

Experimental design

An experimental study was conducted in order to investigate the difference of milk urea among four salt intake levels. It was carried out in the experimental farm Waiboerhoeve (Lelystad, the Netherlands) from 1-2-2010 to 17-3-2010. The first two weeks were adaptation phase for the animals to get used to the new living environment, and from the 3rd to the 6th week was the experimental phase for executing treatments. Sixteen multi-parity Holstein cows were selected with similar body weight (654.9 ± 43.1 kg) and milk production (27.74 ±3.14 kg/day).

A cross-over design was applied for the research. The cows were randomly signed to four treatment groups, and each group corresponds to one treatment sequence. Four dietary treatments were given to the groups in a Digram-balanced Latin Square (Table 1). Roughage and concentrates basis of the feed (see Annex 1) was homogeneous for all four treatments. The difference of diet was in daily salt intake; respectively 0, 0.33, 0.66 and 0.99 kg/cow/day additive dietary salt was mixed with the roughage. Each treatment period lasted 7 days. The first five days were designed as washout days, and it was assumed that the residual effect of the former diet would regress within 5 days. The rest two days were used as test days for data and sample collection. The selected cows were kept in either a tie stall or a free stall. The cows in the tie stall were intensively monitored on, feed intake (kg), water intake (liter), milk production (kg), urine production (kg), milk urea level (mg/dl), plasma urea level (mg/dl) and urinal urea level (mg/dl). Concerning animal welfare issue, only one cow from each treatment group was randomly selected and kept in the tie-stall; the other two cows from the same group were kept in a free stall, whereas these cows were only monitored on feed intake, milk urea and milk production.

Table 1 Four treatment periods and four treatment groups were combined according to a Digram-Balanced Latin Square. The added dietary salt were: Diet 1 = 0 kg/cow/day, Diet 2 = 0.33 kg/cow/day, Diet 3 = 0.66 kg/cow/day, Diet 4 = 0.99 kg/cow/day.

	Treatment Period			
	1	2	3	4
Group A (n=3)	Diet 4	Diet 3	Diet 2	Diet 1
Group B (n=3)	Diet 3	Diet 1	Diet 4	Diet 2
Group C (n=3)	Diet 2	Diet 4	Diet 1	Diet 3
Group D (n=3)	Diet 1	Diet 2	Diet 3	Diet 4

Feeding

In order to drive the cows to eat up all the given feed, each cow received a restricted amount of feed, which was determined as 95% of the maximum eating capacity when the cow was under no feed restriction. All cows were fed 9 times a day from 5:00 to 21:00 o'clock with a 2-hour interval (Annex 2). In the first 8 times feeding, cows received 9.3% feed of the day, and the rest 25% feed was given at 21:00 for overnight consumption. Feed was fully mixed with roughage, concentrates, additive salt and other additive nutrients before giving to the cows. Feeding in tie stall was done manually. In free stall feeding was done by automated feeding system. The feeding machines were accessible for all cows in the free stall, but the feeding system allowed each cow to obtain feed only from one specific, as her own, feeding machine.

Data recording and sample collection

Data and sample collection were performed in the test days (6^{th} and 7^{th} day of every treatment period). In tie stall water intake, feed intake and urine production were recorded manually 9 times a day with 2 hours interval from 5:00 to 21:00 o'clock (Annex 2); same schedule was applied for urine and blood sample collection. In free stall feed intake was recorded whenever the cows visited the feeding machine. For both stall milk samples were collected two times a day during milking from all cows, at 5:00 and 17:00 o'clock respectively.

Laboratory analysis

Urinal urea concentration was analyzed with an enzymatic colorimetric test (Figure 2) provided by urea Liquicolor device (Human®, Max Planck Ring 21, 65205 Wiesbaden, Germany). The principle is that urea produces ammonia during hydrolyzation, and ammonium ions show a green color after reacting with hypochlorite and salicylate. At 578 nm the absorbance of the green color is linearly correlated with the urea concentration in the sample. The regression was calculated by the absorbance of a blank sample (urea = 0 mg/dl) and a standard sample (urea = 80 mg/dl). Based on the linear regression the urea concentration of urine samples were determined.

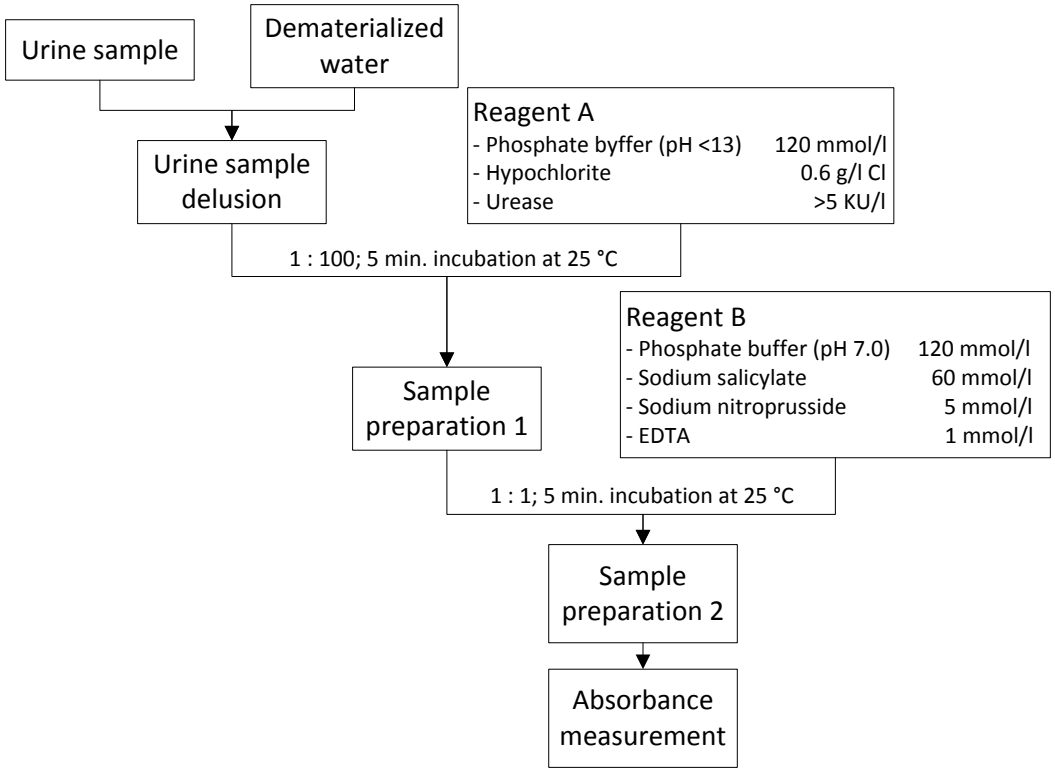

Figure 2 Flow chart of plasma / urinal urea analysis procedure.

Data analysis

The data of feed intake, water intake and urine production of the tie-stall cows were cumulated with every 12-hour recording to calculated half-day records, and the time frame was respectively 5:00-17:00 and 17:00-5:00. The data collected within 5:00-17:00 time frame was

matched with the milk sample being collected at 17:00, namely PM milk data, and the data collected within 17:00-5:00 time frame was matched with the milk sample being collected at 5:00, namely AM milk data. The time frame for calculating half-day feed intake in free stall was the same as that of the tie-stall. However, since there was no fixed feeding time in the free stall, the end feeding time was used as the indicator of feeding time to determine whether the feed intake records belonged to which time frame. The approach of matching feeding records to milk data was the same as the animals housed in the tie stall. Eventually every cow had 4 observations in one treatment period. This dataset was used for ANOVA and linear regression analysis. The mean of the 4 observations were then calculated, and this data set was used for general linear model analysis.

One-way ANOVA was performed in SPSS 16.0 for testing the effect of non-scale independent variables on milk urea and urinal urea: cow group, stall type, treatment period and dietary salt level. Simple linear regression models were performed in SPSS 16.0 for testing the relationship between salt intake, water intake and urine production. Because of unavailability of water intake and urine production data from the free stall, the linear regression test only used the data collected in the tie stall. Proc GLM procedure was performed in SAS® 9.2 for testing the effect of dietary salt on milk urea and urinal urea. According to the basic model of cross over design (Yarandi, unknown publish year; Kotler and Laster, 1997), the first performed model for milk urea prediction was:

Equation 1

$$y_{ijkl} = Intercept + \delta_l + \alpha_{i(l)} + \beta_j + \gamma_k + \beta_k\gamma_k + \varepsilon_{ijkl}$$

Where:

1. y_{ijkl} is milk urea
2. δ_i is the fixed effect due to experimental groups
3. $\alpha_{i(l)}$ is the fixed effect due to cow individuals, which is nested in group
4. β_j is the fixed effect due to dietary salt level (kg/cow/day)
5. γ_k is the fixed effect due to experimental period
6. $\beta_j\gamma_k$ is the interaction between salt level and experimental period
7. ε_{ijkl} is the random error

Because the experimental set up divided the cows into a tie stall and free stall, the stall type may as well affect milk urea. Therefore stall type was also considered as a fixed variable, and the model was formulated as:

Equation 2

$$y_{ijkl} = Intercept + \delta_l + \tau_m + \alpha_{i(lm)} + \beta_j + \gamma_k + \beta_j\gamma_k + \varepsilon_{ijkl}$$

Two variables in this model differed from the previous one:

1. τ_m is the fixed effect due to stall type
2. $\alpha_{i(lm)}$ is the fixed effect due to cow individuals, which is nested in sequence and stall type

Research Results

Progress of the experiment

The experiment was proceeded according to the set up as described previously, but several unexpected events happened during the experimental periods. First, an implementation mistake happened in treatment period 1. According to the experimental set up, the diets for all cows should be homogeneous in contents through all four treatment period except additive dietary salt. However, a certain feed content was not given to group 1, 2 and 3 in period 1, which resulted in a lack of dietary protein, whereas the actual feed content of the feed given in period 1 was not yet known. The mistake eventually lead to an abnormally low milk urea in all cows in these three groups, which can be seen from the mean milk urea in period 1 in table 3 and 4. The abnormal milk urea data had a great influence on the statistical analysis output, and the general linear model was therefore changed in order to achieve the aim research. Second, in the tie stall, the water tap group B was constantly leaking, so that the recorded water intake was remarkably higher than the true consumption.

The data of urinal urea and plasma urea was not yet available due to delayed schedule. According to the plan these data would be used for investigating the relationship between milk urea, urinal urea and plasma urea under different amount of salt intake, and the result was highly important for determining if salt intake could influence urea-N excretion and if milk urea could be used as an indicator of urea-N excretion. Because of the absence of urinal and plasma

data, the discussion in the present study regarding the relationship between milk urea and urea-N excretion was based on literature only, without hard data support.

Feed/water intake and urine/milk production

The feed intake of all individual cows remained relatively constant through all four treatment periods (Figure 3), and neither period (p=0.921) nor diet (p=0.997) had significant effect on feed intake. For water intake, period effect was also insignificant (p=0.072), but it was significantly affected by dietary salt intake (p<0.001). The animals tended to drink more water when more salt in contained in the feed (figure 3). This correlation was tested by linear regression, and the model gave a prediction equation as *water intake = 29.298 (2.016) + 1.003 (0.125) x kg salt intake* (R^2 = 0.527). Urine production was also correlated with salt intake, and the prediction equation was *urine = 9.14 (1.427) + 0.874 (0.088) x kg salt intake* (R^2 = 0.628). However, the correlation between water intake and urine production was relatively low (R^2=0.287). Daily milk production was affected by the dietary salt intake level (Figure 4). With higher daily salt intake, milk production tended to be lower.

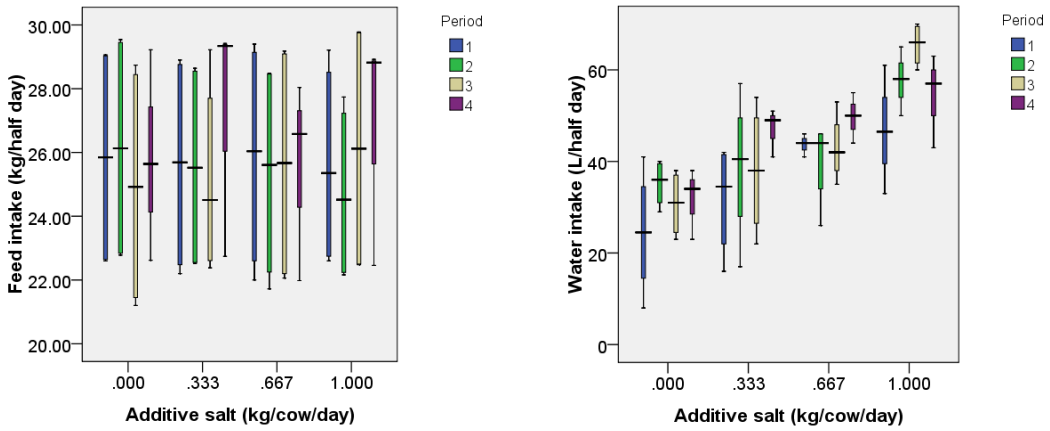

Figure 3 The left figure is the variation of half day feed intake with different dietary salt level, clustered in treatment periods; the right figure is the variation of half day water intake with different dietary salt level, clustered in treatment periods.

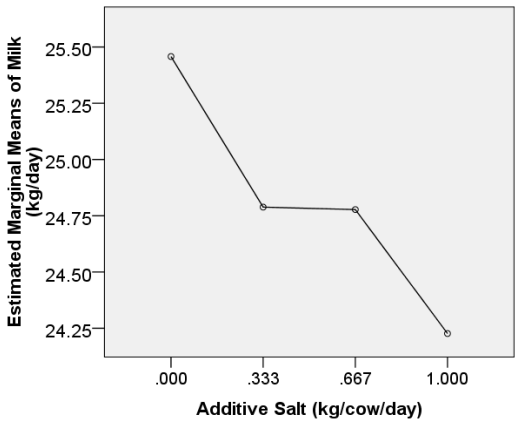

Figure 4 Milk production with different levels of additive dietary salt. The milk production is corrected for cow effect by estimating marginal means.

Milk urea

As mentioned before, the urea mean in period 1 was remarkably lower than other periods (Figure 5). Statistically the milk urea of all cows in period 1 is significantly different from other periods ($p<0.001$), whereas the milk urea in group 2, 3 and 4 are not significantly different from each other ($p=0.938$). The raw data set shows that most milk urea data in group 2, 3 and 4 are rather low (16.85 ± 2.2 mg/dl).

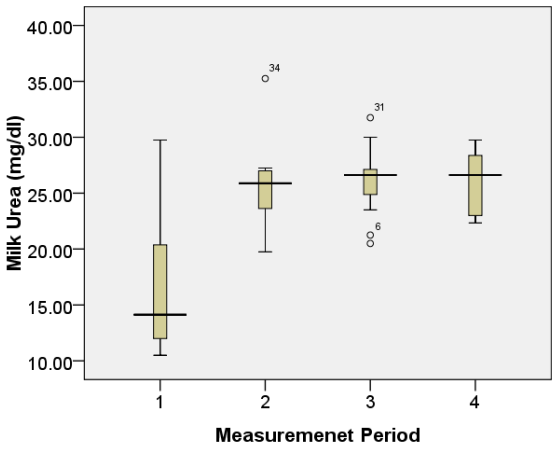

Figure 5 Milk box-plot of milk urea in each treatment periods. The bars present the overall mean and variation of all experimental groups in each period.

Comparing the cows within the same groups, cows in free stalls had relatively higher milk urea mean (Figure 6), but the difference of stall type on milk urea is significant only in group D.

Except group D, all bars seem have extreme values, and 5 of low-value outliers are remarked by SPSS with circles.

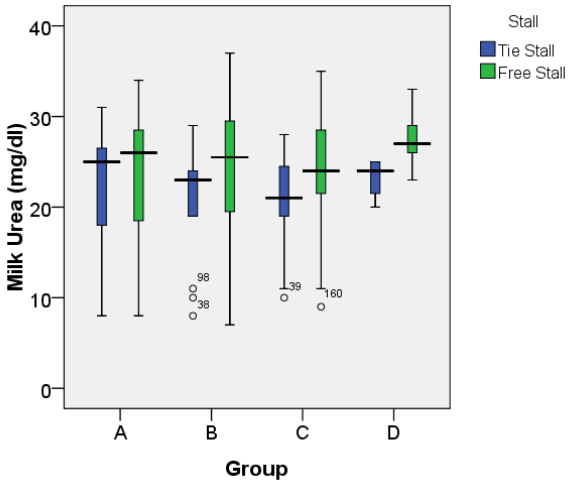

Figure 6 Box-plot of milk urea in each group, and each group is clustered by stall type. The bars present the milk urea mean and variation of all treatment periods.

With the increase of dietary salt level, milk urea showed a down-going trend (Figure 7).

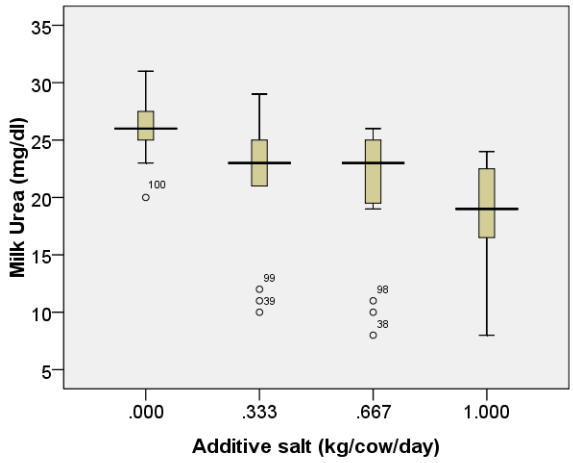

Figure 7 Relationship between milk urea with additive salt level

Looking at the difference in every treatment period, milk urea was mostly negatively correlated with dietary salt (Figure 8). While this trend was different in period 4, that cows fed with 0.999 kg salt/cow/day had a higher milk urea than the cows fed with 0.667 kg salt/cow/day, though the milk urea was close.

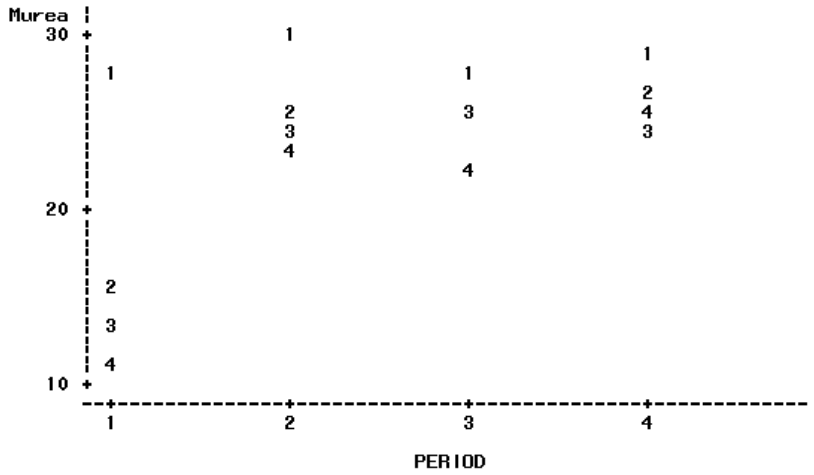

Figure 8 Plot of milk urea level (mg/dl) in the treatment period. The symbol (number) is the dietary salt level, respectively 1 = 0 kg/cow/day, 2 = 0.333 kg/cow/day, 3 = 0.667 kg/cow/day, and 4 = 0.999 kg/cow/day. In period 3, dietary salt level-2 is hidden because of overlapping with dietary salt level-1.

In a general linear model (Equation 1), experimental group, individual cow (nested in group), dietary salt (kg/cow/day), treatment period and dietary salt-period interaction were added as fixed variables in Proc GLM procedure in SAS (Table 2). The dependent variable was milk urea. The model explains 97.6% of the variation in milk urea, and the statistic output shows that all the variables have significant effect on the variation of milk urea. The strongest effect belongs to treatment period, which has the highest sums of squares, and the second strongest factor is dietary salt.

Table 2 Output of Proc GLM procedure in SAS, where milk urea was the dependent variable, and experimental group, individual cow (nested in group), dietary salt (kg/cow/day), treatment period and dietary salt-period interaction were added as fixed variables. R^2 = 0.976.

Source*	DF	Type III SS	Mean Square	F-value	P-value
Group	3	86.036	28.679	17.62	<0.001
Cow (Group)	8	186.863	23.358	14.35	<0.001
Salt	3	423.780	114.260	88.64	<0.001
Period	3	761.929	253.976	156.06	<0.001
Salt * Period	6	118.110	19.685	12.10	<0.001

* Group = experimental group A, B, C and D; Cow = cow individuals of the sixteen sampled cows; Salt = dietary salt intake in 0, 0.333, 0.667 and 0.999 kg/cow/day; Period = experimental period 1, 2, 3 and 4

The general linear model (Equation 2) designed for this experimental setup was also tested by Proc GLM procedure in SAS (Table 3), where milk urea was the dependent variable, and experimental group, stall type, individual cow (nested in group and stall type), dietary salt (kg/cow/day), treatment period and dietary salt-period interaction were added as fixed variables. The statistic output was the same as which of Equation 1, but in this model took stall type into account, and this variable also showed significant effect on milk urea. Stall type separated and explained a part of variation which was expressed by cow individuals in equation 1, whereas there was still remained variation to be explained by cow individuals.

Table 3 Output of Proc GLM procedure in SAS, where milk urea was the dependent variable, and experimental group, stall type, individual cow (nested in group and stall type), dietary salt (kg/cow/day), treatment period and dietary salt-period interaction were added as fixed variables. $R^2 = 0.976$.

Source*	DF	Type III SS	Mean Square	F-value	P-value
Group	3	86.036	28.679	17.62	<0.001
Stall type	1	99.370	99.370	61.06	<0.001
Cow (Group*stall type)	8	186.863	23.358	14.35	<0.001
Salt	3	423.780	114.260	88.64	<0.001
Period	3	761.929	253.976	156.06	<0.001
Salt * Period	6	118.110	19.685	12.10	<0.001

* Group = experimental group A, B, C and D

Stall type = free stall and tie stall

Cow = cow individuals of the sixteen sampled cows

Salt = dietary salt intake in 0, 0.333, 0.667 and 0.999 kg/cow/day

Period = experimental period 1, 2, 3 and 4

Discussion

Experimental design

Crossover study design with a 4x4 Digram-balanced Latin Square set-up was applied in this study. This design has three features regarding design balance (Kotler and Laster, 1997):

1. treatment is balanced with period, as the treatments are applied to the same number of subjects;

2. treatment is balanced with carryover effect (if existing), as each treatment is affected by the carryover effect of other treatments in equal times. Nevertheless, carryover of a placebo treatment, if is applied in the experimental design, will be more frequent;
3. carryover effect (if existing) is balanced with period, as the carryover effect of a treatment is equally occurred in each period.

Based on feature 1, it could be expected that there period factor and the period-treatment interaction would not significantly affect the independent variable. Nevertheless, unexpectedly this expectation does not agree with the statistic output in this study (Table 2). The effect of period and its interaction term should have been disturbed by some other factors which were not included in the model.

Feature 2 and 3 both concern carryover effect, which refers to the situation that the main effect of the previous treatment continues in the following treatment. Usually the carryover effect can be reduced by planning a "washout" period or using particular experimental designs like Digram-balanced Latin Squares. Both measures were applied in this study. The duration of each treatment period was 7 days, and the first 5 days were used as washout period, assuming the residual effect of each diet would regress within 5 days. Nevertheless, in this study it was assumed that the treatment effect would be regressed within 5 days. Carryover effect could be tested by being added as an additional independent variable, and also assume the effect of one treatment would not continue till the second following treatment period. The statistic output suggested that in this study there was no significant effect of the carryover effect (Table 3). Therefore the carryover effect was unlikely a factor that caused significant period effect. Theatrically if carryover effect is absent, the group effect, as receiving the treatment in different sequence, should be insignificant on the dependent variable. But in this study milk urea was under the influence of group. The abnormal urea data in period one might be the cause, because statistically these urea data were considered as the response to the sequence used in period 1.

Table 6 Output of Proc GLM procedure in SAS, where milk urea was the dependent variable, and experimental group, individual cow (nested in group), dietary salt (kg/cow/day), treatment period, dietary salt-period interaction and carryover were added as fixed variables.

Source*	DF	Type III SS	Mean Square	F-value	P-value
Group	3	86.036	28.679	17.62	<0.001
Cow (Group)	8	186.863	23.358	14.35	<0.001
Salt	3	423.780	114.260	88.64	<0.001
Period	3	761.929	253.976	156.06	<0.001
Salt * Period	6	118.110	19.685	12.10	<0.001
Carryover	3	8.431	2.810	0.51	0.6787

* Group = experimental group A, B, C and D

Cow = cow individuals of the sixteen sampled cows

Salt = dietary salt intake in 0, 0.333, 0.667 and 0.999 kg/cow/day

Period = experimental period 1, 2, 3 and 4

Carryover = carryover effect due the previous dietary salt level

Although milk urea could be affected by many other factors, they were unlikely to lead to a large variation in this study. Cows were selected according to several criteria, so the effects of breed (Johnson and Young, 2003), parity (Godden et al., 2001), body weight (Hojman et al., 2005) and milk production (Arunvipas et al., 2003; Johnson and Young, 2003; Hojman et al., 2005) should be restricted. The experiment was conducted within a relatively short period, so seasonal effect (Godden et al., 2001; Arunvipas et al., 2003; Johnson and Young, 2003; Rajala-Schultz and Saville, 2003; Hojman et al., 2005) was limited. Sample and data collection was implemented in a equal frequency in the morning and evening, and the GLM procedure used averaged data for analysis, so time effect (Broderick and Clayton, 1997; Godden et al., 2001) was also avoid.

Feed intake, water intake and urine production

The feed intake of cows did not vary with dietary salt level. The model used in this study assumed that the consumed salt equaled to the amount of salt added in the feed. This assumption was supported by the constant feed intake, which ensured that the designed dietary salt level per cow per day resulted in different levels of true salt consumption (Figure 9).

The positive correlation between salt intake and water intake shown in this study was expected, as well as the positive correlation between salt intake and urine production. The physiological explanation for drinking behavior is that salt in the blood increases osmotic pressure, and that triggers the release of vasopressin from pituitary. On one hand vasopressin leads to thirst and stimulates drinking behavior, so higher water intake. On the other hand, it also promotes sodium excretion via urine. Early study suggested that consumed sodium(Weeth and Lesperance, 1965; Bannink et al., 1999) and the amount of sodium excretion (Kume et al., 2008) was positively correlated to urine production. Since urea is a major content of urine, it is unclear if increase urine production will result in an increased urea excretion. In dairy cattle consumed water is mainly excreted via milk, urine, sweat and breath moisture, so a positive correlation between water intake and urine production could be expected. It was proved in the present study but relatively weak (R^2=0.287).

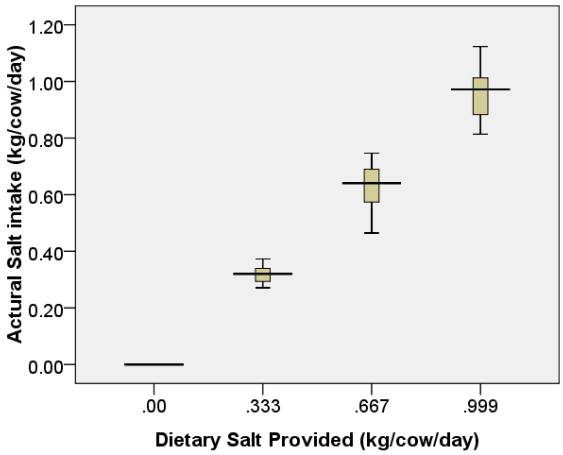

Figure 9 Relationship between actual salt intake and provided dietary salt.

General Linear Model

The feeding mistake happen in treatment period 1 was the only noticed cause of the significant period effect. Skipping the abnormal data in period 1 would result in an unbalanced dataset, and that would increase the complexity of statistical analysis procedure. An alternative measure was to isolate the effect of period 1, and the model was therefore corrected as:

Equation 3

$$y_{ijkl} = Intercept + \delta_l + \tau_m + \alpha_{i(lm)} + \beta_j + \lambda_n + \lambda_n\gamma_k + \beta_j\lambda_n\gamma_k + \varepsilon_{ijkl}$$

Where :

1. y_{ijkl} is milk urea
2. δ^{l} is the fixed effect due experimental group
3. τ_m is the fixed effect due to stall type
4. $\alpha_{i(lm)}$ is the fixed effect due to cow individuals, which is nested in sequence and stall type
5. β_j is the fixed effect due to salt intake
6. λ_n is the effect due to period 1
7. $\lambda_n\gamma_k$ is the interaction between period 1 and other periods; the term equals to the fixed effect due to period other than period 1
8. $\beta_j\lambda_n$ is the interaction between salt intake and period 1
9. ε_{ijkl} is the random error

The model output is given in table 7. The model separated the period 1 effect from other experimental periods, and thereafter period effect, namely the P1*Period term in the model, was no longer significant (p=0.731), and the group effect was also remarkably reduced (p=0.3071). The effect of stall type and dietary salt remained significant.

Table 4 Output of Proc GLM procedure in SAS, where milk urea was the dependent variable, and experimental group, stall type, individual cow (nested in group), dietary salt (kg/cow/day), period 1, period 1-period interaction and dietary salt-period 1-period interaction were added as fixed variables. R^2 = 0. 964.

Source*	DF	Type III SS	Mean Square	F-value	P-value
Group	3	8.149	2.716	1.26	0.3071
Stall	1	99.370	99.370	46.17	<0.001
Cow (Group Stall)	7	87.494	12.500	5.81	<0.001
Salt	3	512.813	170.937	79.43	<0.001
P1	1	760.564	760.564	353.42	<0.001
P1*Period	2	1.364	0.682	0.32	0.7310
Salt*P1	6	99.063	33.021	15.34	<0.001

* Group = experimental group A, B, C and D

Cow = cow individuals of the sixteen sampled cows

Salt = dietary salt intake in 0, 0.333, 0.667 and 0.999 kg/cow/day

P1 = period 1

Period = experimental period 1, 2, 3 and 4

The parameter estimates of intercept, stall type, dietary salt level and period 1 effect are listed in Table 5. For stall type, the milk urea level from tie stall cows was 4.19 mg/dl lower than from free stall. This difference was unexpected because the total given amount and quality of the diet was the same. Lack of movement might be the cause of reduced milk urea level. With no walking, the energy requirement for the tied cows is lower, so tissue breakdown for energy supplication will happen less frequently. Since urea is a metabolite of muscle catabolism, consequently the urea production will be lower when cows do not move.

Table 5 The parameter estimates of intercept, stall type, dietary salt level and period 1 effect. Dependent variable is milk urea (mg/dl).

Parameter[2]		Estimate	SD	t-value	p-value
Intercept		25.89583	0.946928	27.35	<.0001
Stall type	0	-4.1875	1.037308	-4.04	0.0004
Stall type[1]	1	.	.	.	.
Dietary salt	1	5.333333	0.733488	7.27	<.0001
Dietary salt	2	2.64875	0.733488	3.61	0.0012
Dietary salt	3	1.002917	0.733488	1.37	0.1828
Dietary salt[1]	4	.	.	.	.
P1	0	-12.75	1.19778	-10.64	<.0001
P1[1]	1	.	.	.	.

[1] The parameter is set to 0 as reference

[2] Stall type 0 = tie stall, 1 = free stall

Dietary salt (kg/cow/day) 1 = 0, 2 = 0.333, 3 = 0.667, 4 = 0.999

P1 0 = period 1, 1 = non-period 1

Utley et al. (1970) and Burgos et al. (2007) observed that milk urea was lower with a higher water intake. Although the present study focused on the effect of salt intake, the physiological basis should be similar since we also observed that increased salt intake was associated with increases water intake. Lewis and Dahl (1961) observed a positive effect of salt on blood urea. Lewis and Dahl (1961) and Godwin and Williams (1984) found that intraruminal infusion of salt (NaCl) in sheep resulted in a decreased plasma urea level. Since milk urea was highly correlated

with plasma urea (Thornton and Wilson, 1972; Broderick and Clayton, 1997), logical deduction suggests milk urea should also decrease in similar cases.

The quantity of milk urea (mg) was also negatively correlated with salt intake (Figure 10). The amount of excreted milk urea with no additive dietary salt was significantly higher than the amount with 1 kg additive dietary salt ($p<0.001$). This result supported the potential to use milk urea concentration for monitoring actual N excretion. Since the feed intake was relatively constant, it could be assumed that the N intake was constant. The reduced milk urea might be therefore caused by improved N utilization, whereas it was also possible the rest non-milk urea N was excreted through other pathways like urine and feces.

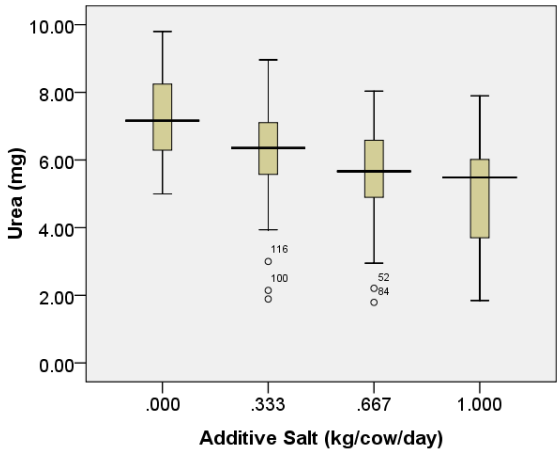

Figure 10 Relationship between quantity of urea and additive salt intake. Urea was calculated as *urea (mg) = milk urea concentration (mg/dl) × milk production (kg) / 200*, assuming 1 kg milk = 1 liter.

Milk urea is only a part of excreted N. In terms of the ammonia emission issue, urinal urea and fecal urea excretion are more important. Zhai et al., (2005) suggested that milk urea was closely related to total N excretion ($R^2 = 0.7$), urinal N excretion ($R^2 = 0.85$) and feces N excretion ($R^2 = 0.22$). Although the present study showed a negative effect of dietary salt on milk urea, it was unclear if urinal N and feces N would be affected in the same way. For using milk urea as an indicator of N excretion, it is also important to know if salt can influence the correlation between milk urea, urinal N and feces N. Moreover, urinal urea is normally measured in concentrations, while the absolute amount of excreted N is unclear. It is therefore also

important to not only look at the concentration of urea in urine but also the quantity. These subjects should be investigated further.

Besides the effect of dietary salt on N excretion, the farming economy should also be considered before using dietary salt in practice. The previous section in the present study observed that higher salt intake was associated with a lower milk production (Figure 4). Due to the limited observations in the present study, it was unclear if the effect of salt on milk production was objective. Assuming that it was, then for reducing N excretion the farmers could either sacrifice a part of milk production or raise more cows to make up the production difference. The former measure would be relatively preferable since the later measure might again result in more excreted N. Nevertheless, to finally determine if it is acceptable to sacrifice production, further economic study is also required.

Conclusion

In this study, milk urea was lower when cows received more dietary salt, while water intake and urine production was higher. Feed intake was not affect by the salt level. Stall type also showed an effect on milk urea, but the reason was not yet clear. Our research result suggests that salt could be used as a feed additive to reduce milk urea. However, to know if salt also decreases urinal N excretion and feces N excretion and if milk urea could be used as an indicator of N excretion in order to monitor ammonia emission, further study is required. The impact of dietary salt on farming economy should also be studied before being applied in practice.

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Annex

Annex 1

Ration based on cow (600 kg) in mid lactation producing 28 litres of milk per day

Raw materials	kg DM per day
Maize silage	15.0
Wheat straw	1.3
Soybean meal (46%)	4.1
Limestone	0.18
Salt	0.08
Urea	0.03
Monocalciumphosphate	0.01
Mervit® Snijmais premix	0.15
Total	20.91
Nutrients	
Crude protein (%/DM)	16.5
VEM (per kg DM)	918
OEB (g/kg DM)	19
DVE (g/kg DM)	89
NDF (g/kg DM)	348
Starch (g/kg DM)	221
Ca (g/kg DM)	6.7
Na (g/kg DM)	2.2
K (g/kg DM)	14.7

Annex 2

Schedule of action in test days

	Milk[1 3]	Feed[1 2]	Water[1]	Urine[1 3]	Blood[3]
5:00	√	√	√	√	√
7:00		√	√	√	√
9:00		√	√	√	√
11:00		√	√	√	√
13:00		√	√	√	√
15:00		√	√	√	√
17:00	√	√	√	√	√
19:00		√	√	√	√
21:00		√	√	√	√

[1] measuring / recording

[2] supplying

[3] sampling