Effective nitrogen preservation during urine collection from Holstein heifers fed diets with high or low protein content

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ABSTRACT

Six Holstein heifers (body weight = 535–625 kg) fed a total mixed ration containing either high protein (13.4%) or low protein (9.0%) were used to evaluate the effect of 3 urine collection methods (chilled, acidified before collection, or acidified after 6 h of collection) on urinary N preservation. In a 2-period crossover design, 16-d diet adjustment stages preceded five 24-h collections. Urinary catheters were inserted 1 d before the collection periods. Urine collection tubes were configured to split urine to 3 collection containers: 1 acidified with 6 N HCl before collection at a rate calculated to reduce pH to below 2, 1 acidified every 6 h during collection to pH below 2, and 1 located in a large cooler of ice. Collection method did not affect urinary concentration of N or urine urea-N (9.2 ± 0.9 g/L and 6.58 ± 0.9 g/L, respectively) or urinary excretion of N or urea-N (82 ± 3.8 g/d and 59.5 ± 3.8 g/d, respectively). These 3 collection methods are equally effective in preserving N during urine collection, but the “chilled immediately” approach may be useful for studies focused on ammonia emission. Urinary and fecal N excretion were significantly different across collection days; fecal N was more highly variable than urinary N. Intake and apparent N digestibility decreased during the collection week, and excretion of urinary and fecal N increased, particularly on d 5. (Stable rectal temperatures suggested no urinary infections.) Improvements in total collection methodology will support continued progress in the understanding of livestock N utilization and post-excretion changes in manure N.

Key words: urine nitrogen, storage, dietary protein, heifer

INTRODUCTION

Various preservation methods have been used to minimize urinary and fecal N volatilization during total collection, with most efforts focused on preserving urinary

N. Nitrogen may be lost during sample collection and storage as a result of urea decomposition and ammonia volatilization. These compounds are a minor part of fecal N (Misselbrook et al., 2005; Sato and Nakajima, 2005), but account for most of the urinary N (Burge et al., 1993; Marini and Van Amburgh, 2005; Colmenero and Broderick, 2006).

In the early 20th century, sulfuric acid was identified as effective in preserving ammonia N in urine of steers (Cochrane, 1915); the author of this work concluded that “figures for total ammonium N are worthless unless special precautions are taken to overcome the rapid ammoniacal decomposition.” Since the time of that observation, several acids (sulfuric, hydrochloric, boric, phosphoric) have been used with success. López et al. (1998) demonstrated that, as long as the acidified urine had similar final pH, sulfuric or hydrochloric acid was equally effective in retaining N in sheep and goat urine. Ferraz et al. (2006) demonstrated that acidification with hydrochloric acid before or after 24-h human urine collection was effective in preserving urinary oxalate, calcium, magnesium, citrate, creatinine, and uric acid.

For unacidified urine samples, temperature during storage is an important consideration. Watson and Duerden (1977) observed that refrigeration at 4°C was effective in preventing bacterial growth in human urine for up to 72 h. Similarly, Porter and Brodie (1969) observed less bacterial growth in samples chilled during shipping (9–15°C, 2–3 d shipping time) than in urine samples shipped without preservation. Samples chilled during shipping yielded similar results to those acidified with boric acid if transit time was 6 h or less, but if chilled samples were delayed in transit, bacterial growth was greater than in acidified samples. Even acidified samples may need to be chilled; human urine samples preserved with boric acid and stored overnight had altered culture results compared with acidified samples analyzed on the day of collection (Gillespie et al., 1999).

Dietary N content can alter the N profile of urine samples, and high-protein diets may challenge preservation methods by increasing urinary N content, especially the concentration of urine urea N. When Holstein heifers were fed diets with increasing dietary

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N content (1.5–3.4%), the urea N content of urine increased from 18 to 79% of total urinary N (Marini and Van Amburgh, 2003). Similarly, in lactating cows almost all of the increase in N excretion with increasing dietary RDP content was in the urine, and urine urea N increased from 68 to 81% of urinary N (Reynal and Broderick, 2005).

Ammonia emissions from manure have become a significant air quality concern because ammonia catalyzes the formation of small particles, leading to the formation of haze. These small particles also have negative human health implications (NRC, 2003). To better understand the effect of diet on ammonia emissions, it is advantageous to collect urine and feces for use in emissions studies. To be applicable, these samples need to remain in a natural state. Given that acidification of the samples traps liberated ammonia in solution, it is not possible to study ammonia emission rates when the samples have been acidified. Therefore, alternative collection methods that maintain the biological activity of the samples are needed.

Duration of collection of feces necessary for accurate and repeatable measurement of digestibility was the subject of numerous publications in the early part of the 20th century. In general, collection periods of at least 7 d were preferred, and collection periods of up to 24 d were used in some studies. The focus of these reports was feces collection; when urinary catheterization is used, the question of duration of collection period is worth revisiting because of the risk of urinary tract infections (Biertuempfel et al., 1981; Warren, 2001).

The objective of this study was to compare the effects of 3 collection methods on N preservation of urine samples from Holstein heifers fed diets that differed in protein content. In addition, the effect of duration of collection period on variability of N excretion data was evaluated.

MATERIALS AND METHODS

Animals and Diets

All animal work was approved by the Virginia Tech Animal Care and Use Committee. Six pregnant Holstein heifers (170 ± 8.7 d in gestation at the start of study; BW 535–625 kg) were fed 2 dietary treatments (Table 1) in a crossover design with two 21-d periods. The treatments were a high-protein (HP) diet (13.4%) and a low-protein (LP) diet (9.0%). Diets with HP supplied 121% of NRC-required protein and LP diets supplied 70% of the required protein (NRC, 2001). Treatment groups were balanced for BW and days pregnant. Heifers were housed in a single group in a freestall barn and fed in Calan doors (American Calan, Northwood, NH)

Table 1. Ingredient and chemical composition of treatment diets¹ fed to 6 Holstein heifers

Item	HP	LP
Ingredient, % of dietary DM		
Corn silage	31.2	31.2
Wheat straw	12.8	12.8
Grass hay	39.6	39.6
Corn grain	0.0	11.6
Soybean meal (48%)	14.4	2.8
Mineral–vitamin mix ²	2.0	2.0
Chemical composition		
DM	60.8	60.7
CP	13.35	9.01
NDF	53.79	53.56
ADF	30.04	29.36
Ca	0.72	0.56
P	0.29	0.32
Mg	0.32	0.28
Ash	7.84	6.74

¹HP = high protein; LP = low protein.

²Contained 19.1% Ca; 6.1% P; 6.4% Na; 10.1% Cl; 2.0% K; 3.5% Mg; 2.0% S; 31 ppm of Cu; 940 ppm of Zn; 840 ppm of Mn; 31 ppm of Co; 64 ppm of I; 20 ppm of Se; 48,351 IU/kg of vitamin A; 88,889 IU/kg of vitamin D; 7.8 mg/kg of vitamin E.

once daily at 1400 h. Heifers were fed for 10% refusals and water was available ad libitum. Feed offered and refused was recorded daily and individual ration ingredients of hay, straw, corn silage, and grain mix were sampled weekly throughout the study.

Heifers were handled regularly, trained to the halter, and adapted to the Calan doors for 1 wk before the start of the study. On d 10 of the first period, all heifers were brought to the metabolism barn for a 24-h acclimation period to evaluate response to confinement to the metabolism stalls. Heifers were calm, with little vocalization or restlessness, and were returned to the Calan doors for the remainder of the diet adaptation period.

Sample Collection

Total collections of urine and feces were conducted in 5-d balance periods following 16 d of diet adaptation. On d 15 of each period, heifers were fitted with a urinary catheter (22 French, 75 mL; C.R. Bard Inc., Covington, GA) and moved into metabolism stalls for a 24-h adaptation period. Daily feed offered and refused was recorded during the balance periods. Feed refusals were sampled by heifer on each day of each balance period. Rectal temperatures were measured daily at 0600 h.

Every 6 h, feces were removed from behind the heifer and placed in 130-L plastic containers and sealed. Feces were weighed, pooled by day, mixed thoroughly, and subsampled every 24 h. For urine collection and preservation, the urinary catheter was connected to

Table 2. Statistical model used to analyze all variables¹

Source	Urine measures		All other measures	
	df	Error df	df	Error df
Treatment	1	10	1	10
Collection method	2	20	NA ²	NA
Treatment × collection method	2	20	NA	NA
Collection day	4	40	4	40
Treatment × collection day	4	40	4	40
Collection method × collection day	8	80	NA	NA
Treatment × collection method × collection day	8	80	NA	NA
Total	179		59	

¹Error terms were interactions with heifer.²NA = not applicable.

approximately 2 m of Tygon tubing (Saint-Gobain Performance Plastics, Akron, OH) that ran from the catheter through a leg band to a sterile urine collection bag (C. R. Bard Inc.) fastened to the stall divider. The top of the urine bag was mounted 1 m from the base of the stall to allow optimum flow (gravity flow) to all 3 collection containers. From the bag, a 10-cm piece of Tygon tubing entered a 3-way splitter. Tubing attached to the outlets of the splitter entered three 20-L polypropylene collection containers via a 10-mm hole in the screw-on caps (drilled to fit snugly around Tygon tubing). Every 6 h, all urine collection containers were weighed, processed as described below, and replaced with clean collection containers.

For the ice treatment, 20-L collection containers were filled approximately 75% with water and frozen. At the start of each collection week, containers with ice (1 per heifer) were removed from the freezer, filled to the rim with cold water, and placed in large foam coolers. A second empty collection container was placed in the cooler adjacent to the ice-filled collection container. Tubing carrying urine destined for the ice treatment was coiled through the water at the top of the ice-filled collection container to improve cooling effectiveness. Urine then flowed into the collection container; level of urine in the final container was always lower than level of ice in the cooling container, facilitating gravity flow.

For the “acid-before” treatment, a preliminary experiment was conducted to determine the expected volume of urine excreted by heifers in 6 h and the quantity of acid needed to reduce pH to <2. Based on the results of that study, 500 mL of 6 N HCl was added to the acid-before collection containers. The final pH of urine in the acid-before treatment ranged from 0.4 to 1.7. The urine in the “acid-after” collection container for each heifer was acidified at the end of each 6-h period according to the urine:acid ratio in the acid-before collection container for that same heifer. Every 24 h, urine was pooled by heifer and storage treatment, pH was

measured, and samples were collected and frozen immediately.

Laboratory Analysis

Feed and feed refusal samples were dried at 60°C to a constant weight and ground through a 1-mm screen in a Wiley Mill (Arthur H. Thomas, Philadelphia, PA). Feed ingredients were analyzed in duplicate for NDF and ADF (Van Soest et al., 1991), total Kjeldahl N, ash, Ca, P, and Mg (AOAC, 1984). Refusals, feces, and urine samples were analyzed in duplicate for total Kjeldahl N (AOAC, 1984). Urine samples were analyzed in duplicate for urea plus ammonia-N (reported as urea N; Kaplan, 1965).

Statistical Analysis

The effects of diets, collection methods (urine measures only), collection days, and their interactions were analyzed using the MIXED procedure of SAS (SAS Institute, 2004) with the model defined in Table 2. A compound symmetry covariance structure for day (subject = heifer × diet) was used in analyzing all data. Orthogonal contrasts were used to evaluate the linear and quadratic effects of day, and the slice option was applied to interactions to determine significance on each day of collection. Results are reported as least squares means, and differences were declared significant at $P < 0.05$. Trends were declared at $P < 0.10$.

RESULTS AND DISCUSSION

Dietary Protein and Utilization of DM and N

The effects of dietary treatment (HP vs. LP) on DMI, DM digestibility, N intake, urine output, urinary N, fecal N, total N excretion, N digestibility, and N balance are shown in Table 3. Dietary protein content had no

Table 3. The effects of treatment (T), collection day (CD), and their interaction on DM and N utilization

Item	Diet ¹			<i>P</i> <		
	HP	LP	SEM	T	CD	T × CD
DMI, kg/d	11.1	10.7	0.32	0.40	0.01	0.01
DM digestibility, %	67.4	66.8	0.87	0.61	0.01	0.01
N intake, g/d	228.9	149.0	5.59	0.01	0.01	0.04
Urine volume, L/d	11.2	9.0	1.99	0.01	0.05	0.01
Urinary N, g/d	110.0	52.9	4.95	0.01	0.01	0.07
Urinary urea N, g/d	89.3	29.6	4.01	0.01	0.04	0.54
Fecal N, g/d	69.2	70.5	3.20	0.79	0.01	0.01
Apparent N digestibility, %	69.6	52.6	1.41	0.01	0.01	0.02
N balance, g/d	49.5	25.5	4.85	0.01	0.01	0.28

¹HP = high protein; LP = low protein.

significant effects on DMI and DM digestibility, as has been observed by others feeding diets similar to these (Gabler and Heinrichs, 2003; Cole et al., 2005; Groff and Wu, 2005). Urine volume was greater in heifers fed the HP diet, likely because greater urine volume was required to excrete the excess N (Sannes et al., 2002). Nitrogen intake, urinary N, and urinary urea N excretion were higher with the HP diet, and fecal N was not affected by dietary protein content. The increase in excretion of urinary N and constant fecal N excretion in response to increased dietary protein content have been reported previously (Marini and Van Amburgh, 2003; Marini et al., 2004). Nitrogen digestibility and N balance were higher in heifers fed the HP diet compared with those fed the LP diet. Increased apparent N digestibility with increasing dietary protein content is likely attributable to dilution of metabolic fecal N.

Collection Method and Urinary N Retention

Collection method had no significant effect on concentration or excretion of urinary total N or urea N (Table 4), and there was no interaction of diet with collection method. Both the acid-before and acid-after methods have been used to preserve urinary N, with acids used including boric acid, hydrochloric acid, phosphoric acid, and sulfuric acid (e.g., Burge et al., 1993;

Wilkerson et al., 1997; Hoffman et al., 2001; Sevi et al., 2006). Often, acidified urine samples are frozen at -20°C before analysis to provide additional control of N loss (e.g., Gehman et al., 2008; Khan et al., 2008). In the current study, the ice, acid-before, and acid-after methods yielded similar urinary N concentrations. The ice collection method avoids the hazard of acid use and would be especially appropriate for studies in which subsequent ammonia volatilization from urine is the explicit focus.

Collection Day and Duration of Collection Period

Interaction of Diet with Day. There was a significant interaction of diet and day for fecal N, and a trend for the same for urinary N ($P < 0.07$). The magnitude of the interaction on urinary N was small, with response to diet diverging on d 2 and 3 (Figure 1). Day slices for urinary N confirmed that the effect of diet was highly significant on each day of collection. In contrast, fecal N, which was much more variable than urinary N, was not affected by the main effect of diet, and the interaction of diet and day was obvious on d 3 (Figure 2a). Fecal N excretion was similar in heifers fed HP and LP diets on d 1, 2, 4, and 5, but slice analyses indicated that heifers fed the LP diet excreted more fecal N on d 3 than did heifers fed the HP diet. A bio-

Table 4. The effects of collection method (CM) on urinary N concentration and total excretion

Item	CM ¹			SEM	<i>P</i> -value ²	
	Acid-before	Acid-after	Ice		CM	CM × T
Urinary N concentration, g/L	9.18	9.25	9.20	0.94	0.97	0.67
Urinary N excretion, g/d	81.3	81.4	82.0	3.77	0.96	0.57
Urinary urea N concentration, g/L	6.53	6.37	6.85	0.93	0.36	0.64
Urinary urea N excretion, g/d	58.7	57.7	62.1	3.78	0.31	0.79

¹Acid-before = collection containers acidified before collection; acid-after = collection container acidified after 6 h of collection; ice = collection container chilled.

²The 3-way interaction CM × treatment (T) × collection day was not significant.

Figure 1. Effect of dietary treatment (high CP = open symbols; low CP = closed symbols) and collection method (acidified before collection, acidified after 6-h collection, or stored on ice) on urinary N excretion by day. (Treatment SEM = 4.95, $P < 0.01$; day SEM = 4.01, $P < 0.01$; diet $\times$ day SEM = 5.68, $P < 0.06$; collection method SEM = 3.77, $P < 0.96$; diet $\times$ collection method SEM = 5.33, $P < 0.57$; diet $\times$ day $\times$ collection method SEM = 7.17, $P < 0.88$.)

logical explanation for this interaction is not obvious. There was no effect of the interaction of diet and day on N balance (Figure 2b).

Effect of Collection Day and Duration of the Collection Period. Intake and digestibility of DM and N declined in a linear fashion during the collection week (Table 5). The decline in feed intake and digestibility implies increasing discomfort associated with confinement to the collection stalls. However, these heifers were accustomed to being handled and the metabolism stalls were large, measuring 1.22 m in width by 1.98 m in length, with rubber-filled mattresses as a surface. Despite these precautions, intake and digestibility declined, particularly on d 5.

Collection day also affected urinary, fecal, and total N excretion (Table 5; Figures 1 and 2a), with excretion of all of these increasing linearly across the collection week (fecal N increased linearly and quadratically). Increases

Figure 2. Effect of dietary treatment (high CP or low CP) on a) fecal N excretion and b) N balance by day. (Fecal N: treatment SEM = 3.20, $P < 0.74$; day SEM = 2.81, $P < 0.01$; diet $\times$ day SEM = 3.60, $P < 0.01$. N balance: treatment SEM = 4.85, $P < 0.01$; day SEM = 4.97, $P < 0.01$; diet $\times$ day SEM 5.94, $P < 0.27$.)

in urinary N were caused by linear increases in urine weight and urinary and fecal N concentration (data not shown). Because of the combination of reduced intake, reduced digestibility, and increased urinary excretion, N balance declined linearly across the collection week.

Significant effects of day implied that one or more days deviated from the mean or that some pattern

Table 5. The effects of collection day (CD) on DM and N digestion and excretion

Item	CD					SEM	P -value ¹		
	1	2	3	4	5		CD	L	Q
DMI, kg/d	11.1	10.9	11.5	10.8	10.4	0.32	0.01	0.01	0.01
DM digestibility, %	70.4	66.8	68.6	66.1	63.1	0.78	0.01	0.01	0.23
N intake, g/d	192	187	199	188	179	5.58	0.01	0.01	0.03
Fecal N, g/d	63	69	70	74	73	2.58	0.01	0.01	0.04
Apparent N digestibility, %	65.3	61.3	62.5	58.7	57.8	1.23	0.01	0.01	0.27
Urine volume, L/d	9.24	10.3	9.97	10.5	10.4	2.00	0.05		
Urinary N, g/d	76	82	79	81	90	4.01	0.01	0.01	0.25
Urinary urea N, g/d ²	56	58	57	58	67	4.17	0.04	0.02	0.16
Total N excretion, g/d	140	151	149	155	163	5.88	0.01	0.01	0.85

¹L = linear effect; Q = quadratic effect.

²Urinary urea N determined as urea N + $\text{NH}_3\text{-N}$.

Table 6. The effects of the length of collection on variability in urinary N (g/d)¹

CD included	SEM			P-value					
	T	CD	CM	T	CD	CM	T × CD	T × CM	T × CD × CM
1 to 5	4.95	4.01	3.77	0.01	0.01	0.95	0.07	0.57	0.90
1 to 4	4.82	3.71	3.61	0.01	0.09	0.78	0.01	0.52	0.66
1 to 3	5.10	3.87	3.87	0.01	0.07	0.52	0.01	0.31	0.62
2 to 5	4.91	3.97	3.81	0.01	0.01	0.95	0.08	0.64	0.70
2 to 4	4.57	3.48	3.48	0.01	0.39	0.72	0.02	0.55	0.27

¹CD = collection day; T = treatment; CM = collection method.

was present in the data. Multiple explanations are possible. If adaptation to the stall or to the catheter was inadequate, the first day's data would be suspect. Increasing the duration of catheterization increases the risk of urinary tract infections, which is more likely to affect latter days in the period (Warren, 2001; Bubenik et al., 2007). However, body temperatures were normal throughout the study and no animals exhibited symptoms of illness during or after collection weeks. Although no negative reaction to the urinary catheters was directly observed, results may have differed if heifers were not catheterized. Similarly, stall comfort would be more likely to affect performance in the latter portion of the collection period, although stall size and design would seem to obviate this concern.

In this study, the later days of the collection period appeared to be more of a concern than the earlier days. To assess this, various days were eliminated from the analysis and the effect of treatment, storage, and day with the resulting collection period were assessed. Collection periods evaluated included d 1 through 5, d 1 through 4, d 1 through 3, d 2 through 5, and d 2 through 4 (Table 6).

For urinary N, the largest reduction in variability came with eliminating the data from d 5. Urinary N excretion on that day was notably higher than in d 1 to 4. A further reduction in variability in urinary N excretion was achieved with the elimination of d 1 data, suggesting that a longer precollection adaptation to the stall and catheter was desirable. Additional work is needed to determine whether the effect of day is caused by factors biasing the results on the first and last day of the collection or whether this variation is random "noise."

Fecal N was much more variable than urinary N (Figure 2), but changing the number of collection days used to analyze fecal N data did not alter the significance of treatment, collection day, and their interaction (Table 7). Using data from only d 1, 2, and 3 yielded the lowest SEM for treatment and day, but the change in SEM for any combination of days was not noticeably different. Firm conclusions about optimum duration

of total collection of feces and urine cannot be drawn from this data set. One possibility worth exploring is whether variability in urinary N continues to increase with increased duration of collection. If so, the most accurate measures of urinary N may call for a shorter collection period than is needed for measurement of fecal N.

CONCLUSIONS

Urinary N preservation was similar for urine acidified at the start of collection, acidified after 6 h of collection, or chilled immediately, regardless of dietary protein content. No interaction was observed between dietary protein and urine preservation method, so any of these 3 collection methods is appropriate and the chilled immediately approach may be useful for studies focused on ammonia emission. Urinary N excretion was more consistent among days than was fecal N excretion, with increasing duration of collection increasing variability of both measures. Additional work is needed to more fully evaluate optimum duration of collection of feces and urine for N balance measurements.

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Table 7. The effects of the length of collection on variability in fecal N (g/d)¹

CD included	SEM		P-value		
	T	CD	T	CD	T × CD
1 to 5	3.20	2.54	0.79	0.01	0.01
1 to 4	3.06	2.46	0.47	0.01	0.03
1 to 3	2.40	1.88	0.20	0.01	0.01
2 to 5	3.77	2.87	0.78	0.04	0.01
2 to 4	3.77	2.86	0.45	0.05	0.02

¹CD = collection day; T = treatment.

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