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BRIEF COMMUNICATION: Nitrogen constituents in urine spot samples from sheep grazing standard and hyper-diverse pastures

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Abstract

This study investigated the effect of pasture and management system on nitrogen (N) pools related to N excretion in spot samples of urine in ewes grazing a hyper-diverse (>18 sown plant species) versus standard (perennial ryegrass + clover) pasture, managed under either contemporary (industry best-practise) or regenerative (high residuals, longer rotation length) management over a two-year period (Mar 2023-Dec 2024). A total of 160 Romney ewes were randomly allocated to four self-contained farmlets including standard pastures with contemporary management (Std-Con), standard pastures with regenerative management (Std-Reg), diverse pastures with contemporary management (Div-Con), and diverse pastures with regenerative management (Div-Reg) treatments. Urine samples were collected seasonally over two years. Results show that estimated urine volume was significantly (P=0.05) higher in ewes on diverse pastures (Div-Con: 5.19 L/d; Div-Reg: 4.97 L/d) compared to standard pastures (Std-Con: 3.79 L/d; Std-Reg: 3.52 L/d). Total urinary N output, (Purine Derivative: Creatinine ratio (PDC index), or the ratios of N, uric acid, allantoin, and urea to creatinine were not significantly affect by treatments. However, most parameters varied significantly across sampling times (P<0.01), indicating temporal effects related to physiological stage and seasonal variation in pasture quality. Overall, diverse pasture systems tended to support greater urine output and more consistent microbial protein supply (PDC index). Urinary metabolites were measured from a single urine spot sample collected within an existing grazing study and caution is still needed when using this method to avoid inaccuracies in estimating urinary metabolites due to variations between individual animals and studies.

Keywords: creatinine; grazing system; purine derivatives, regenerative management

Introduction

Pastoral farming systems have the potential to influence environmental outcomes in grazing. Incorporating diverse pastures with legumes and herbs has been shown to enhance nitrogen (N) use efficiency in sheep (Woodmartin et al. 2024). More recently, regenerative management has gained attention as a strategy to further improve environmental sustainability (Grelet et al., 2021). A holistic evaluation of these approaches is therefore crucial to determine their potential in reducing N losses from grazed pastures.

Assessing N utilisation requires knowledge of urinary N excretion, purine derivatives (PD) and creatinine to evaluate microbial protein supply (MPS) (Faichney et al. 1995; Makkar and Chen 2004). While total urine collection provides accurate data, it is costly, labour-intensive and impractical under grazing conditions (Silva et al. 2018).

Spot urine sampling is an alternative technique using urinary creatinine to estimate urine volume and PD excretion as a proxy for MPS (Chen et al. 1995; Makkar and Chen 2004) This approach assumes that creatinine excretion is primarily dependent on muscle mass and liveweight (LW) data (David et al. 2015; Chizzotti et al. 2008) and is generally diet independent (Lee et al. 2019; Park et al. 2025). By combining spot sampling with animal LW, it is possible to calculate a creatinine excretion factor (CEF) then estimate total urine volume (Tebbe and Weiss 2018). Once urine volume is estimated, urinary N and PD excretion can be derived, allowing researchers to evaluate both N utilisation efficiency and N losses in grazing systems.

The aim of the study was to use urine spot samples collected within an existing grazing study to evaluate whether the addition of legumes and herbs to a perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.) pasture under conventional or regenerative management would influence N utilisation including MPS in sheep grazing systems, using spot urine samples as a proxy for protein status and N excretion.

Materials and methods

This study was conducted from March 2023 to November 2024 as part of a larger study established in 2022 at Whenua Haumanu sheep farmlets located at the Pasture and Crop Research Unit, Massey University, Palmerston North, Manawatū, New Zealand (40°23'24.3"S 175°36'24.5"E). Experimental procedures were approved by the Massey University Animal Ethics Committee (MUAEC22/17) and conducted in accordance with the Massey University Code of Ethical Conduct.

Mature Romney ewes (n=160; 67.25±10.92), between 3-6 years of age, were blocked by LW before being randomly assigned to treatments including standard pasture with contemporary management (Std–Con), standard pasture with regenerative management (Std–Reg), diverse pasture with contemporary management (Div–Con) and diverse pasture with regenerative management (Div–Reg). Contemporary management followed the Beef + Lamb NZ industry grazing guidelines for sheep (Beef + Lamb NZ, 2018) and best-practice use of chemicals and mineral fertilisers to optimise pasture performance, while regenerative management involved having longer grazing intervals, greater post grazing residuals, and minimal chemical and mineral fertiliser use (Grelet et al. 2021).

At each sampling date, six ewes per treatment (n=24 total) were randomly selected for urine collection. Sampling occurred across key management dates: mating (March 2024), scanning (June 2023/2024), set stocking (August 2023/2024), docking (October 2023), and weaning (November 2023/2024) over 2023-2024. Ewes were removed from pasture and yarded, and urine was collected between 9:00-11:30 a.m. using transitory apnoea (Benech et al., 2015). Samples were collected into 70 mL containers, with one subsample acidified (pH 4.0) using concentrated sulphuric acid and another left untreated. All samples were stored at -20 °C for later analysis. Total N was measured by the Dumas combustion method (AOAC 968.06, Leco Corporation, St Joseph, MI, USA, 1994). Creatinine, uric acid, and allantoin were determined colourimetrically (Henry et al., 1974; Fossati et al. 1980; O'Dell 1993). Ammonia in acidified samples was analysed via the phenol-nitroprusside method (Marshall et al. 2020).

Creatinine excretion factor determination

The CEF was estimated after reviewing published literature (Table 1).

Table 1. Summary of the studies used to determine the urinary creatinine excretion factor (CEF) in sheep[1].

Study	Reference	Country	Breed	Time	Unit[2]	n[3]
1	(Hovell et al., 1987))	UK	Not specified	<1 year	mg/kg $LW^{0.75}$	9
2	(Lindberg and Jacobsson, 1990)	Sweden	Swedish Landrace	<1 year	mg/kg $LW^{0.75}$	4
3	(Chen et al., 1991)	UK	Romney	>1 year	mmol/kg $LW^{0.75}$	4
4	Dewhurst and Webster (1992)	UK	Clun Forest × Welsh	>1 year	mg/kg $LW^{0.75}$	16
5	(Dapoza et al., 1999)	Spain	Rasa Aragonesa	>1 year	mmol/kg $LW^{0.75}$	45
6	(Liu and McMeniman, 2006)	Australia	Merino crossbred	<1 year	mg/kg $LW^{0.75}$	28
7	(Dipu et al., 2008)	India	Muzaffarnagari	>1 year	mmol/kg $LW^{0.75}$	8
8	(David et al., 2015)	Brazil	Texel × Ile de France	<1 year	mmol/kg $LW^{0.75}$	16
9	(Silva et al., 2018)	Brazil	Hair sheep	<1 year	mg/kg of $LW^{0.75}$	12

[1]Urinary creatinine concentration (UCC) and corresponding LW in sheep were identified via Google Scholar (https://scholar.google.com) using the search terms: "sheep urinary creatinine", "sheep urine", and "urine volume". Only studies published in peer-reviewed journals were retained. [2]Units were standardised to mg/kg metabolic live weight ($LW^{0.75}$) to ensure consistency across studies. [3]Total number of animals.

Estimation of urine volume

Urine volume (L/day) was estimated by applying the equation as described by Pacheco et al. (2009), with CEF (mg/kg $LW^{0.75}$), animal's metabolic live weight ($LW^{0.75}$) and UCC (mg/L) and urinary N excretion (g/day) was calculated as urine volume (L/day) multiplied by urine N (%).

$$Urine\ volume_{\ L/day} = \frac{CEF_{mg/kg\ LW} \times LW^{0.75}}{UCC_{mg/L}}$$

Prediction of microbial nitrogen supply

A proxy for microbial N supply (MNS) was estimated using the purine derivatives to creatinine ratio (PDC index) (Totty et al. 2013b; Chen and Gomes 1992). The urinary excretion of purine derivatives is considered an indicator of microbial protein synthesis in the rumen (Valadares et al. 1999) and was calculated as PD total = [Allantoin (mmol/L) + Uric acid (mmol/L)], using the following equation:

$$Purine\ derivative\ (PDC) index = \frac{PDtotal\ (\frac{mmol}{L}) \times LW^{0.75}}{Creatinine\ (\frac{mmol}{L})}$$

Statistical analysis

The CEF was estimated using the PROC Mixed in SAS 9.4 (SAS Institute Inc., Cary, NC, USA), treating study as a random effect. To account for variation in study precision, the inverse of the number of observations (n) was used in the WEIGHT statement (Wang and Bushman 1999; St-Pierre 2001). Nitrogen-related parameters were analysed similarly, with pasture treatment and animal ID as fixed effects, sampling time as a repeated measure, and year as a random effect. Statistical differences were declared at P<0.05 using Fisher's Protected Least Significant Difference (LSD). Data normality and homogeneity of variance were assessed with PROC Univariate.

Results and discussion

Analysis of reviewed literature showed that sheep over 1 year of age had a significantly higher least squares mean CEF (51.67±1.88 mg/kg $LW^{0.75}$) compared to that of lambs up to one year old (23.28±1.65 mg/kg $LW^{0.75}$) ($P<0.01$; Figure 1). As all samples in this study were from sheep over 1 year of age, the corresponding CEF value was used for subsequent calculations.

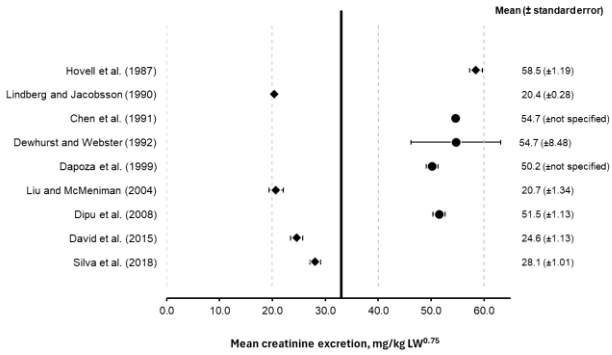

Figure 1. Summary of reported mean creatinine excretion values (± standard error) from the literature, expressed as mg/kg $LW^{0.75}$. The central vertical line represents the average across all studies. Diamonds indicate data from growing lambs up to 1 year of age, while circles represent sheep > 1 year of age.

Ewes grazing diverse pastures (Div-Con and Div-Reg) had lower concentrations of N, creatinine, uric acid, allantoin, and urea than those on standard pastures (Std-Con and Std-Reg; Table 2). Estimated urine volume was significantly higher in ewes on diverse pastures (Div-Con: 5.19 L/d; Div-Reg: 4.97 L/d) compared to standard pastures (Std-Con: 3.79 L/d; Std-Reg: 3.52 L/d; Table 2). Treatments did not significantly affect total urinary N output, PDC index, or the ratios of N, uric acid, allantoin, and urea to creatinine (Table 2). However, most parameters varied significantly across sampling times ($P<0.01$), indicating temporal effects related to physiological stage and seasonal variation in pasture quality (Table 2; Figure 2).

Table 2 Urine composition (concentration) in spot samples of urine from ewes grazing diverse (Div) and standard (Std) pastures (P) under contemporary (Con) and regenerative (Reg) management in five sampling times (T) between 2023 and 2924. Values are LSM ± (SEM).

Measurement[1]	Treatments[2]					*P*-value		
	Std-Con	Std-Reg	Div-Con	Div-Reg	SEM	Treatment	Time	P×T
Nitrogen (%)	0.78^{b}	0.45^{b}	0.25^{a}	0.36^{ab}	0.067	< 0.01	< 0.01	< 0.01
Creatinine (mmol/L)	2.24^{b}	2.20^{b}	1.06^{a}	1.89^{b}	0.336	< 0.01	< 0.01	0.08
Uric acid (mmol/L)	0.97^{b}	0.93^{b}	0.56^{a}	0.93^{b}	0.077	< 0.01	< 0.01	0.03
Allantoin (mmol/L)	5.88^{c}	5.71^{c}	2.77^{a}	3.77^{b}	0.616	< 0.01	< 0.01	0.05
Urea (mmol/L)	150.16^{c}	137.12^{c}	78.26^{a}	114.09^{b}	15.863	< 0.01	< 0.01	< 0.01
Estimated Urine volume (L/d)	3.79^{a}	3.52^{a}	5.19^{b}	4.97^{b}	0.525	0.05	< 0.01	0.09
Urinary N (mg/d)	8.46	8.34	7.82	8.52	0.735	0.81	< 0.01	0.56
PDC index	81.9	88.1	79.11	82.06	7.590	0.79	< 0.01	0.03
Nitrogen:Creatinine	0.21	0.21	0.23	0.21	0.216	0.64	< 0.01	0.19
Uric acid:Creatinine	0.48	0.50	0.54	0.51	0.136	0.64	< 0.01	< 0.01
Allantoin:Creatinine	2.94	3.08	2.93	2.75	0.304	0.74	< 0.01	0.12
Urea:Creatinine	73.33	71.12	76.93	78.78	4.104	0.50	< 0.01	< 0.01

[1]PDC index =Purine derivative: creatinine ratio. [2]Standard contemporary (Std-Con), standard regenerative (Std-Reg), diverse contemporary (Div-Con) and diverse regenerative (Div-Reg). Replication (n= 48 samples per pasture system). [abc]Superscripts within row are statistically different from each other's by Fisher protected least squared means ($P<0.05$).

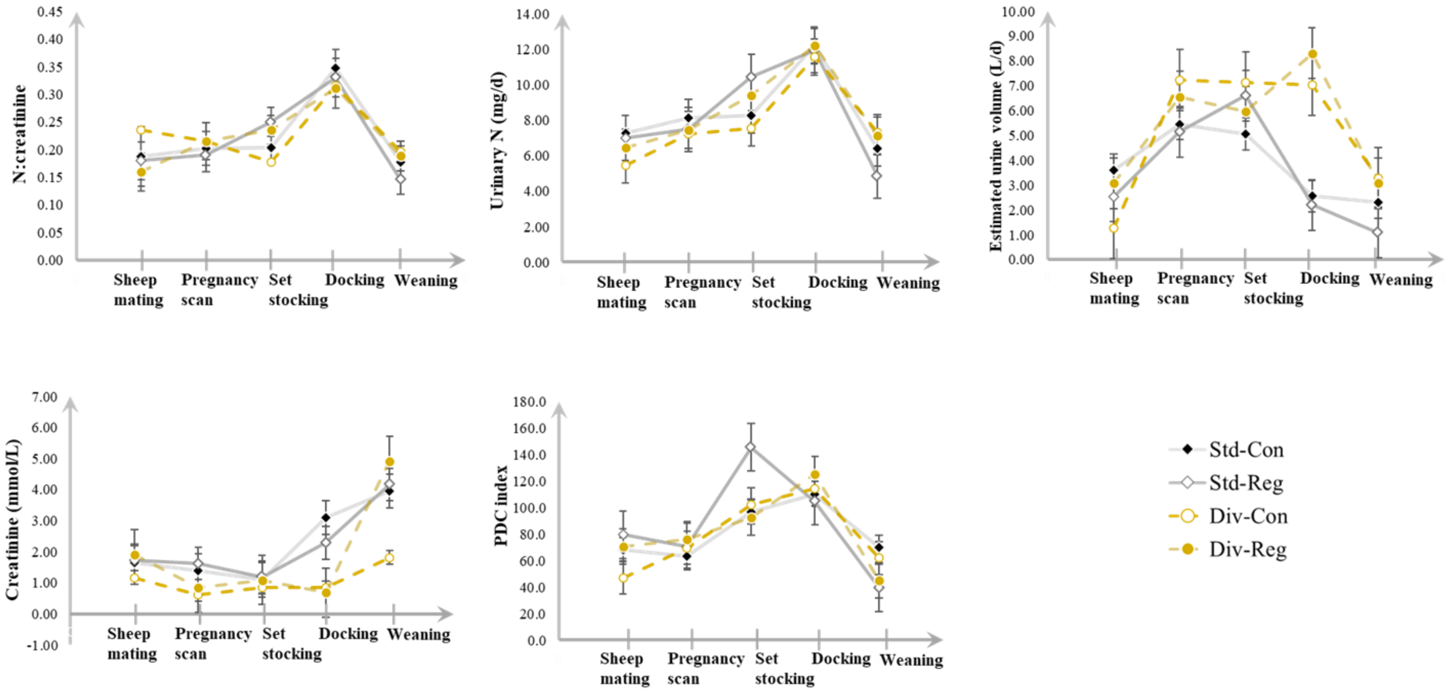

Figure 2. Urinary indicators: Purine derivative to creatinine ratio (PDC_index) and estimated_urine volume (L/d) in spot samples taken from ewes across five sampling dates in 2023-2024: sheep mating (2024 only), pregnancy scan, setstock, docking (2023 only), and weaning, under four pasture systems: standard contemporary (Std–Con), standard regenerative (Std–Reg), diverse contemporary (Div–Con), and diverse regenerative (Div–Reg). Values are least square means (LSM) ± standard error of the mean (SEM).

Urinary N dynamics across sampling times were influenced by both physiological stage of the ewes and pasture system, with trends reflecting seasonal variation in pasture quality (Giltrap and Mc Neill 2020; Litherland et al. 2002). During docking (October), a period typically associated with high pasture growth and quality in New Zealand (Baars 1982; Li et al. 2001), ewes exhibited the highest N:creatinine ratios and urinary N output across all treatments, indicating elevated dietary N intake and excretion. This is comparable with spring pasture peak, particularly in protein-rich species like clover, with studies reporting a crude protein (CP) content of 22.6 (Litherland et al. 2002), and 20.35 (Giltrap and Mc Neill 2020) in ryegrass-white clover pastures.

While measuring total urine output was not an objective of this study, estimating urine volume was essential for assessing the quantity of urinary N among treatments. Estimated urine volumes followed a similar trend, peaking during pregnancy scanning (June) and docking, especially in ewes on diverse pastures (Div-Con and Div-Reg), suggesting higher water intake or dilution due to lusher, moisture-rich forage. Previous studies have shown increased urine volumes in sheep when fed diets containing chicory or plantain compared to perennial ryegrass-white clover pasture (O'Connell et al. 2016; Woodmartin et al. 2024). Increased urination frequency due to high intake of water, potassium and sodium in chicory diets has shown to result in dilution of components in the urine (Mangwe et al. 2019). Similar results were observed with dairy cows fed plantain having higher urine volumes, resulting from high water content in plantain (Minnée et al. 2020) and lower urinary N including urinary creatinine concentrations (Nguyen et al. 2022). In this study, creatinine concentrations were highest at weaning, likely reflecting decreased urine volume and thus higher solute concentration coinciding with reduced pasture quality in late spring/summer (Litherland et al. 2002). Decreased urine volume is associated with high concentration of urinary solutes (Mangwe et al. 2019).

The PDC index, a proxy for microbial nitrogen supply (Valadares et al. 1999), peaked at set stocking (August) for Std-Con and remained relatively high for the diverse systems through docking. Having a high-quality pasture, broader range of pasture species including plantain has been proposed to increase the rumen digestible protein (Mahboobi et al. 2023; Minnée et al. 2020; Totty et al. 2013a). This suggests that pasture quality and rumen fermentation conditions may have supported enhanced microbial activity in late winter and early spring.

However, data presented here was based on a single spot sample from ewes per season and the daily estimated urine volume was determined using a CEF extrapolated from previous studies with lambs up to one year old having a lower CEF of 23.28±1.65 mg/kg LW0.75 compared to 51.67 mg/kg $LW^{0.75}$ for adult sheep. Due to variations between individual animals and studies (Park et al. 2025; da Silva Júnior et al. 2021), the measurements in this study including urinary metabolites and urinary N suggest a need for caution and need further investigation. Assuming the CEF was known and consistent over a 24-hour period, uncertainties linked with estimated urinary volume are avoided, giving defensible estimates when the N:creatinine ratio is used (Waghorn et al. 2019). Nonetheless, in grazing systems, urine spot sampling technique is still useful to examine dietary effects on urine output (Lee et al. 2019).

Overall, diverse pasture systems tended to support greater urine volume output and more consistent microbial protein supply (PDC index), particularly under contemporary management, with lower urinary N concentrations. These findings align with expected seasonal shifts in pasture nutritional quality, where peak availability of legumes and herbs during spring boosts N intake and excretion, while leaner periods reduce N loading in urine.

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