The *in vivo* digestibility and creatinine excretion pattern of lambs fed plantain (*Plantago lanceolata* L.) and perennial ryegrass (*Lolium perenne* L.)

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Hannah Fletcher

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Abstract

The loss of nitrogen from sheep-grazed pastures can have negative effects on the environment via leaching of nitrate and nitrous oxide emissions. To be able to accurately measure or estimate nitrate leaching and nitrous oxide emissions it is important to be able to quantify urine output from livestock, however this is difficult to do with grazing sheep. This research was carried out with two main research objectives. The first was to investigate the creatinine excretion pattern of sheep fed perennial ryegrass (*Lolium perenne L.)* or plantain (*Plantago lanceolata L.*) at different times of the day. Creatinine is a nitrogenous compound only excreted in urine. It has been used to predict urine volume, accurately with cattle but there is a large amount of unexplained variation when using this method with sheep. The time of day has been proposed as a potential factor of creatinine concentration which may affect the accuracy of predictions.

The second objective was to measure the *in vivo* digestibility of these two forages with sheep. Digestibility of the two forages (especially plantain) has not been studied extensively *in vivo* in New Zealand for sheep and older digestibility data may not be relevant to modern sheep. It was hypothesised that plantain will have a lower DM digestibility *in vivo.*

An 8-day indoor metabolism trial (3-day adaptation period and 5-day collection phase) was conducted using eleven castrated Romney-cross wether lambs. The lambs used were split into two groups (Plantain (n=5) and Ryegrass (n=6)) that were balanced by liveweight. Urine samples were taken from the lambs at 6am, 9am, 12pm, 3pm and 6pm (4 x 3-hour intervals and 1 x 12-hour interval) over 4 days and analysed for concentration of creatinine. Further, the total collection method was applied and feed, feed refusals, faeces and urine were all weighed and sampled to determine in vivo digestibility.

Forage treatment had no influence on the amount of creatinine excreted by sheep or the digestibility of dry matter, ash, fat, nitrogen, neutral detergent fibre, acid detergent fibre and gross energy ($p>0.05$), but did affect creatinine concentration and lignin digestibility ($p<0.05$). The measured lignin digestibility was high for both forages and greater for the plantain than the perennial ryegrass (76.7% > 43.8%, $p<0.05$). This suggests lignin may not be a good indicator of DM or NDF digestibility. The time of day affected both

creatinine concentration and the amount of creatinine excreted per hour ($p<0.05$). Therefore, to improve the accuracy of urine volume predictions identifying a time when creatinine concentration in the urine of sheep is consistently representative of the mean daily creatinine concentration may be beneficial. The results of this experiment suggest this period might be in the early afternoon during the 12pm-3pm period (approximately 6 hours after the lambs were given fresh feed) as this period had the closest mean creatinine concentrations to the daily mean.

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Abbreviations

ADF	Acid detergent fibre
ADG	Average daily gain
AOAC	Association of Official Agricultural Chemists
B+LNZ	Beef + Lamb New Zealand
CH_4	Methane
CO_2	Carbon dioxide
CP	Crude protein
DCE	Daily creatinine excretion
DE	Digestible energy
DM	Dry matter
DMD	Dry matter digestibility
DMI	Dry matter intake
FCR	Feed conversion ratio
GE	Gross energy
GHG	Greenhouse gas
GWP	Global warming potential
IPCC	Intergovernmental panel on climate change
LW	Liveweight
ME	Metabolisable Energy
MfE	Ministry for the Environment
MPI	Ministry for Primary Industries
N	Nitrogen

N_2	Nitrogen gas
NDF	Neutral detergent fibre
NH_4^+	Ammonium
N_2O	Nitrous oxide
NO_3^-	Nitrate
OM	Organic matter
OMD	Organic matter digestibility
RDP	Rumen degradable protein
UNFCCC	United Nations Framework Convention on Climate Change

1 Introduction

Urinary excretion of nitrogen (N) from livestock farming systems is a major source of both nitrate leaching and nitrous oxide (N_2O) emissions (Ledgard et al., 2009). Nitrate leaching can result in the pollution of waterways which poses a threat to water quality, freshwater ecosystems, and human health. Nitrate leaching from livestock is also a source of N_2O emissions. In New Zealand, N_2O emissions account for approximately 9.4% of gross Greenhouse Gas (GHG) emissions and 15.9% of agricultural emissions (MfE, 2025).

In New Zealand sheep farming is pasture-based with more than 95% of feed demands from sheep being met with pasture (Morris, 2013). There are about 24 million sheep farmed on a total of about 4.1 million hectares in New Zealand (StatsNZ, 2021a, 2021b). Perennial ryegrass (*Lolium perenne* L.) and white clover (*Trifolium repens* L.*)* are commonly used for sown pastures in sheep farming systems. However, alternative forages such as plantain (*Plantago lanceolata* L.) are becoming more prominently used by farmers. Plantain is a forage of interest because it has been reported to reduce urinary N excretion from both cattle (Nguyen et al., 2022) and sheep (O'Connell et al., 2016). The inclusion of plantain into a mixed herb-clover sward with chicory, red clover and white clover has also been found to support greater lamb growth pre-weaning (Hutton et al., 2011) and post-weaning (Kemp et al., 2013). However, the digestibility of these two forages for sheep in New Zealand has not been studied extensively *in vivo* with current forage cultivars and sheep genotypes. This is especially true for plantain, which is a relatively novel forage used in sheep farming and has undergone significant genetic change in response to artificial selection with many cultivars having been developed (Judson et al., 2018).

The volume of urine excreted by sheep is an important factor of both nitrate leaching and N_2O emissions, so for effective mitigation strategies to be developed, urine volume has to be taken into consideration. Measuring urine output in a grazing scenario is difficult, so alternative approaches have been proposed. One approach to estimate urine volume involves the use of creatinine in urine spot samples (Maheswaran et al., 2022).

Creatinine is a nitrogenous compound produced in a non-enzymatic anhydration reaction from creatine (Kashani et al., 2020). Creatinine excretion is largely dependent on muscle mass and liveweight (Cetinkaya et al., 2006; Chizzotti et al., 2008; David et al., 2015) and is solely excreted in the urine. In theory, if the amount of creatinine excreted daily is known or can be estimated, it is possible to estimate urine volume from the creatinine concentration in a urine spot sample. This has been done with good accuracy in dairy cattle (Susmel et al., 1995; Valadares et al., 1999).

A few equations to predict urine volume from creatinine concentration from sheep urine have been developed (Jonker et al., 2021; Maheswaran et al., 2022). However, attempts to estimate urine volume from creatinine concentration in sheep urine have not been as successful with large amounts of variation in predicted urine volume (Maheswaran et al., 2022). This variation in predicted urine volume is largely the result of differences in the creatinine concentration of urine spot samples. It may be possible to reduce this variation and make estimations of urine volume more accurate if variables such as forage type and the time of day when samples are taken are investigated for effects on creatinine excretion. Subsequently the objectives of this study were to investigate creatinine excretion at different times of the day in lambs fed plantain (*Plantago lanceolata* L. cv. Agri Tonic) or perennial ryegrass (*Lolium perenne* L. cv. Maxsyn) to understand the excretion pattern of creatinine with a focus on estimating daily urine output, and to measure the *in vivo* digestibility of plantain and perennial ryegrass fed to lambs using metabolism crates.

2 Literature Review

2.1. New Zealand sheep industry

Modern New Zealand sheep farming is heavily focused on meat production and approximately 95% of the produced carcass weight is exported annually (MIA, 2022). New Zealand mostly utilises a pasture-based system for the farming of ruminant livestock where pasture is the main source of feed (contributing over 95% of the total feed) (Morris, 2013).

The number of sheep in New Zealand has dramatically decreased from the historical peak of ~70 million sheep in the 1980's to around 24.4 million sheep in 2023 (Figure 2.1). Approximately 21.4 million sheep were slaughtered over the 2022/2023 production year to produce 436.9 million kg of graded carcass weight (MPI, 2024). Over this same time a combination of increased lamb carcass weights and lambing percentages mean the level of production has only decreased by 14% (B+LNZ, 2022).

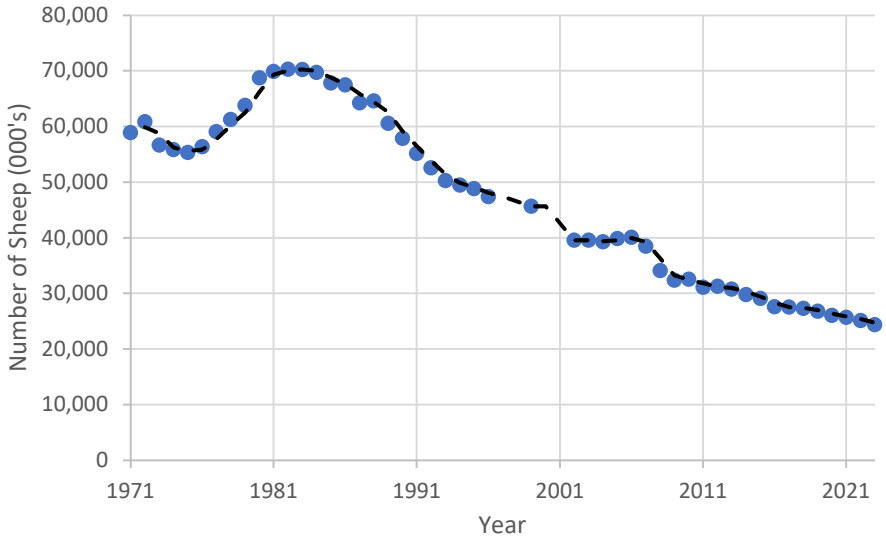


Figure 2.1. Numbers of sheep farmed in New Zealand from 1971-2023. (StatsNZ, 2021b, 2024a). The blue dots represent the number of sheep each year whereas the dashed line represents the trend.

2.2. Environmental impacts

Sheep farming can have adverse effects on the environment which are further exacerbated by the intensification of sheep farming due to higher stocking rates and increased fertiliser usage. Examples of environmental impacts include the emissions of

greenhouse gases (GHG), nutrient leaching, and run-off. These impacts threaten the environment, wildlife, social licensing and future of farming; thus, it is of high priority to mitigate negative effects. For this review, focus will be placed in nitrous oxide (N_2O) emissions and nitrogen (N) leaching.

2.2.1. Nitrous Oxide (N_2O)

Greenhouse gases are gases that contribute to the greenhouse effect by absorbing or re-emitting infrared radiation in the atmosphere. They can be of natural or anthropogenic origin (UNFCCC, 1992). By reducing the heat lost to the atmosphere, GHGs maintain global temperatures that can sustain life and thus are essential to life on earth. However, large increases in GHGs in the atmosphere following the industrial revolution have caused high levels of accumulation beyond historical precedents which has resulted in increasing global average temperatures (Myhre et al., 2014).

Nitrous oxide is one of the three main GHGs relevant to agriculture. It has a potent global warming potential 265 times that of carbon dioxide (CO_2) according to the 5th Intergovernmental Panel on Climate Change (IPCC) assessment report and long lifetime in the atmosphere of 121 years (Table 2.1).

Table 2.1. Atmospheric lifespan and global warming potential (GWP) of GHGs relevant to agriculture (Armour et al., 2021)

Common Name	Chemical Formula	Lifetime in atmosphere (years)	GWP100	
			5th Assessment Report	6th Assessment Report
Carbon Dioxide	CO_2		1	1
Methane	CH_4	12.4	28	27 (non-fossil)
Nitrous Oxide	N_2O	121	265	273

Nitrous oxide is a by-product of nitrification and denitrification pathways carried out by bacteria in waterways and soil. Nitrification involves the aerobic oxidation of ammonium (NH_4^+) to nitrate (NO_3^-) whilst denitrification is the anaerobic reduction of nitrate to nitrogen gas (N_2). Nitrous oxide is produced as a by-product of nitrification and as an

intermediate of denitrification (Klein et al., 2008). The conversion of NH_4^+ to N_2 by nitrification and then denitrification is shown by the following equation.

$$NH_4^+ \rightarrow NO_3^- \rightarrow N_2$$

Emissions of N_2O from agriculture can be either direct or indirect. Direct emissions are the result of nitrification and denitrification of NH_4^+ from N fertilisers, manure, dung and urine in soil. Indirect emissions are from N that has been leached into waterways or NH_4^+ that has been volatised and released into the atmosphere (Klein et al., 2008). In New Zealand, 81% of agricultural N_2O emissions are direct whilst 19% are indirect (MfE, 2024).

In 2022, sheep, beef and grain farming collectively were responsible for 44% of New Zealand's N_2O emissions (3,046 kt CO_2-e) and 29% of New Zealand's total GHG emissions (23,282 kt CO_2-e) (Table 2.2).

Table 2.2. N_2O and gross GHG emissions from New Zealand and the agricultural sector by industry (StatsNZ, 2024).

		ktCO_2e	
Sector	**Industry**	**N_2O**	**Total GHG**
Agriculture		6434	43646
	Horticulture	72	854
	Sheep, beef cattle, and grain farming	3046	23282
	Dairy Cattle farming	3150	18617
	Poultry, deer, and other livestock	167	893
NZ Total		6887	79749

2.2.2. Nitrogen Leaching

Table 2.3. Indirect N2O emissions from agriculture in New Zealand in 1990 and 2022 (MfE, 2024)

Indirect N2O emissions (agriculture)	kt CO_2-e		%	
	1990	2022	1990	2022
Atmospheric Deposition from managed soils	642.7	745.1	61.9	60.8
Nitrogen leaching and run-off	396.1	479.5	38.1	39.2
Total	1038.8	1224.6	100.0	100.0

Nitrogen leaching refers to the movement and loss of N in soil to waterways and groundwater. This can lead to various environmental problems such as impaired water

quality, eutrophication, disruption of ecosystems and contributes to indirect N_2O emissions (Wang & Li, 2019). Sources of N leaching include N fertilisers, urine, dung and effluent (Klein et al., 2008). In New Zealand, N leaching and run-off from agriculture contributed to just under 40% of indirect emissions in 2022 (Table 2.3).

The levels of N leaching in New Zealand sheep farms are not reported to the same extent as GHG emissions; and variation also comes from non-animal factors such as soil type and fertiliser application rate (Bryant et al., 2011), pasture species (Carlton et al., 2019; Maxwell et al., 2019) and timing of N fertiliser application. Other sources of variation such as stocking rate (Monaghan et al., 2005) and stock class (Vérité & Delaby, 2000) exist as well.

Nitrogen leaching involves leaching of both nitrate and ammonia, but nitrate is the major concern due to being leached at a much greater rate (Dymond et al., 2013). A wide range of nitrate leaching has been reported in New Zealand with rates between 0.37-539.7 kg N/ha/year (Table 2.4), however most leaching rates from sheep farms are below 100 kg N/ha/year. The highest leaching rates are associated with high levels of N input such as fertiliser or stocking rate (urine deposition). Of the values in Table 2.4 the only ones to exceed 100 kg N/ha came from a study which applied rates upwards of 200 kg N fertiliser/ha which is abnormally high and not applicable to most New Zealand farms. As of 2021, the National Environmental Standards for Fresh Water have placed a 190 kg N/ha cap on all synthetic N fertiliser application, so fertiliser rates above 200 kg N/ha are not likely to be an issue (MfE, 2021).

The study by Hoogendoorn et al. (2017) from which the highest values were attained from also used mini-lysimeters of the following dimensions 300mm long and 150mm diameter across. The limited depth of these lysimeters may have affected the results as movement of solutes and water in deeper soil is not necessarily the same as in shallower soil. Essentially, in relation to the depths N is leached in soil, these lysimeters only measure a fraction of that movement (Watkins & Shepherd, 2013).

Table 2.4. Nitrate-N Leaching Rates Reported for Sheep in New Zealand.

Method and Class of sheep	Fertiliser N Application kg N/ha/year	NO_3-N Leaching kg N/ha/year	Reference
Dry ewes, with increasing levels of N fertiliser applied and stocking rates. Measurements with mini-lysimeters.	0	103	(Hoogendoorn et al., 2017)
	100	97	
	200	128	
	300	238.3	
	400	324.3	
	500	490.3	
	750	539.7	
Hill Country Farm. Urea applied onto pasture.	0	1.7	(Parfitt et al., 2009)
	300	30.3	
Unknown class of sheep grazed.	144	5.8	(Ruz-Jerez et al., 1995)
	152	7.3	
	400	41.1	
Mole and Pipe system. Ewes stocked at a rate of 14 ewes/hectare. Grazing four different forages. Intensive sheep farming management conditions.	31.8	0.37-0.46	(Maheswaran et al., 2023)
	37.8	0.45-0.75	
	101.3	1.49-9.68	
	55.75	0.37	
	51.55	6.35	
	23	3.51	
Range	0 - 750	0.37 - 539.7	
Range (excl. N fertiliser levels >190 kg N/ha)	0 - 152	0.37 – 103	

2.3. Urine output and accounting for N_2O emissions and N leaching.

Urinary output is a major source of both N leaching and N_2O emissions in the agricultural industry. The amount of urine N deposited onto pasture has a significant effect on nitrate leaching (Di & Cameron, 2007). It has been reported that for some dairy cattle-grazed pastures in Ireland, urine contributed 70-90% of the total N leached (Ledgard et al., 2009). Thus, the deposition of urine is important when estimating the extent of N_2O emissions. Further variation in N_2O emissions from sheep comes from differences in the volume and number of urination events as well. More frequent and smaller urination events can result in greater N_2O emissions than sheep excreting the same urine volume and amount of N that urinate more at a time and less frequently (Marsden et al., 2016).

2.3.1. New Zealand methodology for direct N_2O from sheep in the GHG inventory.

The current methodology used by the New Zealand Government in the national GHG inventory to account for direct N_2O emissions from sheep utilises an equation incorporating population data including numbers of sheep per class per month, N_2O emissions factors and land data (Pickering et al., 2024). Essentially, the amount of N excreted in both urine and faeces per month is estimated per sheep class (e.g. ram), this is then multiplied by the total number of sheep in that class per month. Emission factors are then used to estimate the N_2O emissions as a fraction of N excreted. Emission factors vary on whether N was excreted in faeces or urine and the slope of the land it was excreted on.

The equations currently in use were derived from a meta-analysis of N partitioning studies with livestock where faeces and urine were collected (Pacheco et al., 2018). The following equations for N excreted in urine and direct N_2O from sheep urine are generalised forms and the values 0.005 and 0.0008 represent the emission factors of urine N that is emitted as N_2O on flat or steep slopes respectively.

$$Total\ N\ excreted\ in\ urine\ = Population \times N\ excreted\ in\ urine\ per\ day\ \times days$$

$$N_2Odirect_{U,sheep,t} = \frac{44}{28} \times (Total\ N\ excreted\ as\ urine \times (Proportion\ of\ urine\ N\ excreted\ on\ flat\ or\ low\ slopes\ \times 0.005 + Proportion\ of\ urine\ N\ excreted\ on\ medium\ or\ steep\ slopes\ \times 0.0008)$$

Notably, the methodology used by the government does not consider N concentration or urine volume which can affect N_2O emissions via changes in urine deposition (Marsden et al., 2016). From a convenience and ease of calculation point of view this is a logical choice, but it could misrepresent N_2O emissions from sheep with more or less concentrated urine. Emissions factors for extensively farmed sheep are also smaller than those from intensively farmed sheep (Marsden et al., 2018), however this is partially accounted for by the difference in emission factors for sheep from flat or steep slopes.

Table 2.5 shows the amount of N excreted daily by sheep in a limited number of New Zealand trials. Nitrogen excreted daily in urine ranges from ~3.8-32.9 g N/d whilst N concentration varies around 0.07% to 1.05% (Table 2.5).

Table 2.5. Urine volume, N concentration and daily excretion from New Zealand sheep studies.

Reference	Sheep Used	Method	Urine N Concentration (%)	Urine Volume (L)	Urine N Excretion (g N/d)
(Al-Marashdeh et al., 2020)*	Coopworth ram lambs (8 months old)	Airway Obstruction	0.57 - 0.61	2.9-3.5	17.8-19.7
(McGusty, 2017b)	Romney ewe lambs (5 months)	Metabolic Crates		3.1-3.8	
(Lindsay, 2016)	Romney ewe lambs (5 months)	Metabolic Crates	0.298 - 0.497	2.5-3.3	9.8-12.5
(O'Connell et al., 2016)	Romney ewe lambs (6 months)	Metabolic Crates		3.0-3.5	
(Jonker et al., 2015)	Romney Wethers	Metabolic Crates			9.2-20.8
(Hoogendoorn et al., 2010)*	Romney Ewes	Airway Obstruction	0.36 - 1.05		
(Maheswaran, 2023)*	Ewes	Airway Obstruction	0.07-0.8	0.7-15.2	3.8- 32.9

*Studies using airway obstruction have estimated urine volume and urine N excretion

Using the equations used to calculate N_2O emissions from New Zealand for the national GHG inventory and values of 9 and 20 g N/d, the N excreted per day by individual sheep on flat or low slopes is estimated to be between 0.07-0.16 g N_2O/d compared with 0.011-0.025 for medium or steep slopes which gives a broad range of 3.1-42.9 g CO_2-e per day per sheep (Table 2.6).

Table 2.6. Estimates of daily direct N_2O emissions from sheep urine using NZ GHG inventory methodology.

	Scenario 1	Scenario 2	Scenario 3	Scenario 4
N excreted per day (g N/d)	9	9	20	20
Slope	Flat or low	Medium or steep	Flat or low	Medium or Steep
Direct N_2O from urine (g/d)	0.070714	0.011314	0.157143	0.025143
Direct N_2O as g CO_2e/d*	19.305	3.0888	42.9	6.864

*N2O emissions were converted to CO2-e by multiplying by 273, the Global Warming Potential of Nitrous Oxide according to the 6th assessment report.

2.3.2. Total collection method of urinary output.

Total collection methods refer to methods that involve the collection of all faeces and urine from an animal, usually in a confined space. First, there is an adaptation phase where animals are enclosed in a controlled area (not necessarily in crates) and are given

the test diet. After the adaptation phase, the collection phase takes place when measurements are made. This approach is what was used to develop some of the equations in the NZ GHG inventory (Pacheco et al., 2018) and commonly used in digestibility trials as the difference between the feed eaten and faeces excreted is assumed to represent the digestible matter of a feed as follows:

$$Apparent\ Nutrient\ Digestibility\ (\%) = \frac{Nutrient_{intake} - Nutrient_{in\ faeces}}{Nutrient_{intake}} \times 100$$

By directly measuring and sampling all the excreta, this method provides accurate results. Most of the time total collection is primarily carried out by housing animals in specialized crates ("metabolism crates"), however this method can also be used alongside respiration chambers that measure Greenhouse Gas (GHG) emissions such as methane.

Measurements from the total collection of excreta are thought to be accurate as they are direct measures of what comes out of the animal. However, animals are usually tested for multiple days (commonly 4-7 days) (Farenzena et al., 2017) to improve the data reliability because variation in dry matter intake due to stresses such as isolation can occur (Llonch et al., 2016). For digestibility trials it is also important that multiple days are tested because feed particles can remain in the rumen of sheep for 20-50 hours (Laredo & Minson, 1973). The adaptation period helps reduce the potential impact of stress on dry matter intake and the long reticulo-rumen retention time of forages during the collection phase.

Although the total collection method is considered the most accurate method, it is expensive and has significant labour costs. The ability to apply results from total collection methods to free-grazing animals can also be limited. Isolation stress is known to be able to affect dry matter intake (Llonch et al., 2016), which can go on to affect numerous variables such as digestibility, urine volume and urine N concentration (David et al., 2015).

Urine is often collected using collecting funnels, but catheters are sometimes used. The use of collecting funnels requires animals to be housed in metabolism crates as urine is

collected by funnelling the excreted urine into a container (McDonald, 1958). While catheters require the technical skills and equipment needed to set them up. Both collecting funnels and catheters can be used to directly measure urinary volume and to find urinary concentration values.

2.3.3. Predicting urinary output from creatinine excretion

The limitations of the total collection method have contributed to the development of alternative means of measuring or estimating urinary output. One such alternative method uses daily creatinine excretion and spot-samples of urine. Total creatinine excretion is relatively constant, which is why it has been used to estimate traits of urinary excretion (Kashani et al., 2020). Urine spot samples can also be easily collected by briefly obstructing the airways of sheep (Benech et al., 2015). The method effectively uses the daily creatinine excretion of an animal (known or estimated) and a urine spot sample analysed for creatinine to estimate urine volume, which can then be used alongside N concentration to estimate N excretion. Estimations are made using established equations developed in trials where the total collection method was used. This has already been accomplished with good accuracy for dairy cattle however variance can be underestimated (Susmel et al., 1995; Valadares et al., 1999).

This method holds a lot of promise because animals are not detained for long periods when using this method, so more animals can be tested in a set time frame allowing for a broader scale with less labour per animal. However, the measurements made are not direct, requiring estimates that use established equations or relationships between creatinine excretion and urinary excretion traits. So, the estimation of urinary excretion using creatinine will never be able to fully replace the total collection method when it comes to urinary outputs but could be used to decrease the costs of measuring urinary outputs with reduced stress experienced by the animal.

The following table includes a small number of equations to estimate daily creatinine excretion and urine volume in sheep (Table 2.7). It is of note that the main and only variable of the daily creatinine excretion equations is liveweight (LW), the relationship between liveweight and creatinine is discussed in section 2.5. Creatinine concentration has also been used to predict urine volume.

Table 2.7. Published equations for estimating Daily Creatinine Excretion (DCE) and daily urine volume (L) from sheep.

Reference	Sheep Used	Value Estimated	Equation
(Brody & Lardy, 1946)	Ewes	Daily Creatinine Excretion (mg)	$12.7\ LW^{0.896}$
(Langlands, 1966)	1 year old wethers	Daily Creatinine Excretion (mg)	1.825 LW + 305
(Langlands, 1966)	Ewes	Daily Creatinine Excretion (mg)	1.825 LW + 232
(Field et al., 1974)	Ewes	Daily Creatinine Excretion (mg)	18.16 LW + 93.14
(Dos Santos et al., 2018)	Ram Lambs	Daily Creatinine Excretion (mmol/d)	19.82 BW
(Maheswaran et al., 2022) (Using data from (McGusty, 2017b))	Ewe lambs	Urine Volume	(-1271.4 x creatinine concentration (mmol/L)) + 6289.9
(Jonker et al., 2021)	Ewes	ln Urine Volume (L/d)	5.474-0.8718 ln creatinine concentration (mg/L) + 0.01663 LW
(Maheswaran et al., 2022)	-	Urine Volume (L/d)	$\frac{(\text{mg Creatinine/kg}LW^{0.75}\text{ per day} \times LW^{0.75})}{\text{Creatinine Concentration (mg/L)}}$

2.4. Creatinine

Creatinine is a nitrogenous organic acid produced in the kidneys, liver, and pancreas as a waste product. It has a molecular weight of approximately 113g/mol and is produced from creatine and creatine phosphate via an irreversible non-enzymatic anhydration reaction (Kashani et al., 2020). During this reaction water is removed from creatine or creatine phosphate and the molecule becomes cyclical (Figure 2.2). Creatine phosphate is a short-term energy source that is commonly found in muscle tissue (Salazar, 2014) which is produced from three amino acids: arginine, glycine and methionine (Narayanan & Appleton, 1980).

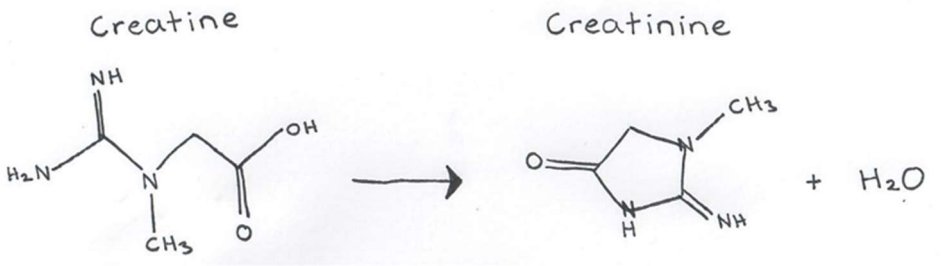


Figure 2.2. Simplified equation of the conversion of creatine to creatinine.

2.5. Factors that affect creatinine excretion

2.5.1. Metabolic Liveweight

Sheep excrete about 0.2-0.5 mmol of creatinine per kg metabolic liveweight ($LW^{0.75}$) per day (Table 2.8). Creatinine excretion is heavily dependent on muscle mass and largely independent of diet (Salazar, 2014). Strong positive relationships between metabolic liveweight (indicative of muscle mass) and creatinine excretion have been reported previously (Cetinkaya et al., 2006; Chizzotti et al., 2008; Tebbe & Weiss, 2018). In one study, liveweight accounted for 70% of the variation in creatinine excretion by growing dairy heifers (Chizzotti et al., 2008), whereas little variation is produced by the diet (Dapoza et al., 1999; David et al., 2015; Susmel et al., 1995; Valadares et al., 1999), the number of meals (Chen et al., 1992) or feed intake (Cetinkaya et al., 2006; David et al., 2015).

The importance of developing sheep-specific equations is demonstrated in Table 2.8, because the creatinine excretion rate in terms of mmol/kg liveweight for sheep is often less than half that of cattle. Del Valle et al. (2023) reported creatinine excretion from multiple previous studies and these values ranged from 0.194 to 0.504 mmol/kg $LW^{0.75}$. This range also encapsulates the values from other studies with sheep shown in Table 2.8.

Table 2.8. Creatinine Excretion Rate (mmol/kg $LW^{0.75}$) of cattle and sheep.

Species	Breed	Class	Diet	Daily creatinine excretion rate (mmol/kg $LW^{0.75}$)	Reference
Cattle	Holstein	Lactating Dairy Cow	Corn silage, pelleted cottonseed hulls and concentrate	1.07	(Chizzotti et al., 2008)
	Holstein	Lactating Dairy Cow	Corn silage and concentrate	1.05	(Chizzotti et al., 2008)
	Holstein	Dairy Cow	Total Mixed Ration	1.33	(Tebbe & Weiss, 2018)
	Holstein	Dairy Bull	Elephant-grass silage and cottonseed hulls	0.98	(Chizzotti et al., 2008)
	Yerli-Kara crossbreds	Bulls	Wheat straw and compounded feed (95% VFI)	1.09	(Cetinkaya et al., 2006)
Sheep	Dorper	Male lambs	50% sorghum silage and 50% concentrate	0.43	(Dos Santos et al., 2018)
	Border Leicester-Merino x Poll Dorset	Female lambs	Oaten Straw, lucerne and legume seeds	0.2	(Yu et al., 2001)
	Texel x lle de France	One year old lambs	Pearl millet or Italian Ryegrass	0.21	(David et al., 2015)
	Santa Inês	Male Lambs	50% hay and 50% concentrate	0.36	(Del Valle et al., 2023)
	Santa Inês	3-month-old lambs	Cynodon hay and 300g/animal concentrate	0.50	(Del Valle et al., 2023)
	Santa Inês	Adult sheep	50% hay and 50% concentrate	0.20	(Del Valle et al., 2023)
	Santa Inês	8-9 month old ewes	Guinea Grass Hay	0.40	(Del Valle et al., 2023)
	Santa Inês	Adult sheep	50% hay and 50% concentrate	0.23	(Del Valle et al., 2023)

2.5.2. Level of feed intake

Creatinine excretion is usually unaffected by feed intake. Wethers fed lucerne and sorghum-based diets at varying levels of maintenance (0.5, 1.0 and 1.5- times maintenance) had similar creatinine-N excretion values (Liu & McMeniman, 2006). Similarly, feed intake has been reported to have no effect on creatinine excretion rates

from lambs fed Italian ryegrass and pearl millet (David et al., 2015). Whilst feed intake has no known direct effect on creatinine excretion it is important to note that feed intake could affect liveweight and muscle mass so it may indirectly affect creatinine excretion if sufficient differences in liveweight are consequently produced.

2.5.3. Protein and non-protein N intake

Total creatinine excretion is largely independent of diet factors such as protein and non-protein N intake even though it is derived from arginine, glycine and methionine. For lactating dairy cattle, increasing urea inclusion in the diet had no effect on creatinine output (Susmel et al., 1995). Protein intake has also been reported to not have a significant effect in cattle (Kertz et al., 1970). The same absence of an effect was found in sheep when pregnant and lactating ewes were fed diets with varying amounts of protein, no significant effect of protein intake was observed on creatinine excreted (Dapoza et al., 1999)

2.5.4. Feed type

In most cases, feed type has not been found to produce an effect on creatinine excretion, however with some feed types there have been differences reported. The effect of feed type in these studies is not always a direct result of the diet but can be a result of a difference in liveweight response to feed type. An example of a diet effect via liveweight on creatinine excretion in sheep is starvation where creatinine excretion was reduced both during the starvation period (7 days) and for a time after returning to normal feed intakes because liveweight decreased due to food deprivation (Van Niekerk et al., 1963). Because feed type can have an indirect effect on creatinine excretion via liveweight, it is important that studies take liveweight and liveweight responses to feeds in consideration when examining creatinine excretion so that direct diet effects are not inferred when liveweight is the key factor.

When feeding pearl millet and Italian ryegrass to one year old lambs, no significant difference was reported in creatinine excretion (David et al., 2015). This agrees with Spanghero (2002) who found creatinine excretion over 6 hours to be unaffected by feeding various types of hay to dry cows. However, the inclusion of legume seeds into a lucerne and oaten straw diet given to 6 month old female lambs increased total

creatinine excretion rate. The diets of the lambs given legume seeds, as well as lucerne and oaten straw, had greater amounts of metabolizable energy and digestible organic matter on a per kg dry matter (DM) basis (Yu et al., 2001) which may have resulted in different liveweight responses, or may be a direct effect of the diet. The results of Yu et al. (2001) can likely be partially explained by differences in liveweight response to the feed offered because the group of lambs fed the control diet of lucerne and oaten straw without legume seeds which had the lowest creatinine excretion rate had the lowest liveweight by at least 2kg by the end of the 8-week trial; whereas the groups supplemented with different legume seeds had similar liveweights and creatinine excretion values.

It cannot be assumed that liveweight differences can explain all creatinine excretion variation in response to different diets though. Diet dependent differences in creatinine-N excretion have been reported between wethers fed varying combinations of lucerne, oaten chaff and sorghum, but not for ewe lambs of the same breed, approximate age and liveweight (Liu & McMeniman, 2006). Similarly, it has been observed that urinary outputs can be underestimated for sheep fed low dry matter forages and that the creatinine excreted per kg liveweight may not be constant between forages (Jonker et al., 2021). This is why it is important that potential relationships between specific feeds and creatinine excretion are investigated and considered when using creatinine as a marker to estimate urinary excretion traits.

2.5.6. Time of day

Whilst production of creatinine is likely to take place at a constant rate due to the spontaneous nature of the reaction which removes water from creatine and lack of regulatory enzymes (Kashani et al., 2020); time of day has the potential to be a factor of creatinine excretion because creatinine is only excreted in urine and the time of day can affect urination frequency and volume (Maheswaran et al., 2022). However, the effect of the time of day on creatinine excretion patterns has not been studied to a large extent in sheep.

In dairy cattle, creatinine excretion rate appears to be relatively unchanging. Creatinine excretion rate doesn't change significantly throughout the day in dairy cattle with a lack

of difference in rate between 5am to 5pm. More creatinine was excreted from 5pm to 5am in this trial, but only by 5%, and this was attributed to urine loss during milking (Valadares et al., 1999). Estimations of daily creatinine excretion from urine collected from dairy cows over 6, 9, 12, 15, 18, or 21 hours were all similar and close to the actual 24 hour excretion value measured (Chizzotti et al., 2008). The amount of creatinine excreted is largely unchanging, but the concentration can vary with lower concentrations reported in the late afternoon at 3pm and 6pm (Cosgrove et al., 2017).

Fewer studies have focused on sheep, but it has been found using sheep with catheters that creatinine excretion rate was similar over 12 and 24 hour periods, though variation was observed when comparing 6 hour periods (Hodgen et al., 1967). This agrees with another study where 4 month old castrated Dorper sheep fed sorghum silage and concentrate and found creatinine excretion rate itself did not vary greatly throughout the day when comparing 4 hour periods in a 24 hour period. Though the ratio of urea to creatinine was higher during diurnal periods than in nocturnal periods and the concentration of creatinine in urine was greater at night (Dos Santos et al., 2018). This supports the idea that creatinine excretion rate is relatively constant throughout the day, but the concentration and ratio of creatinine to other urinary compounds such as urea can change. Thus, it appears creatinine excretion is relatively constant in sheep, which is similar to cattle, however the studies mentioned here all used total collection methods, and the method may have an impact.

Creatinine excretion measured from spot samples has been a lot more variable. For example, it is important that the timing of events such as feeding are considered when taking spot samples. When taking spot samples from fistulated male sheep 0, 2, 4, 6, and 8 hours after feeding to predict creatinine excretion and urinary volume, estimates from 4 and 6 hours after feeding were significantly different to the observed values (Vieira et al., 2022). With spot samples, often creatinine concentration is what is measured and when applying an equation to estimate urine volume using creatinine concentrations from spot samples, Maheswaran et al. (2022) found a wide and unrealistic range of values for urine volume estimations, with values of 40L/d being estimated. If possible, time periods in which creatinine excretion rate is more representative of the daily mean should also be identified via studies of creatinine concentration in sheep to improve the

accuracy of predicting urinary excretion. Similar work that tried to predict daily urinary purine derivative output using the ratio of purine derivatives to creatinine in dairy steers has been done by Chen et al. (1992), who suggested that when using the ratio of purine derivatives to creatinine to predict daily urinary purine derivative output, urine samples should be taken between 3pm to 8pm.

2.5.7. Summary of factors

In summary, liveweight is the most influential factor affecting creatinine excretion with limited diet effects. Factors of a singular feed such as feed or protein intake do not appear to significantly affect creatinine. However, large amounts of variation in creatinine concentration have been reported and estimated urine volume from low DM forages often tends to be underestimated. Thus, forage type and time of day are also relatively unknown factors when it comes to creatinine excretion in sheep.

2.6. Forages

Creatinine excretion is largely independent of forage type, but it is important that the absence of an effect is verified, or any effects identified are accounted for. When investigating creatinine excretion for the purpose of estimating urinary volume and concentration, there are two groups of forages of greater interest. The first group of forages are those which are commonly used in sheep farming such as perennial ryegrass. The second includes forages which are known to influence urinary excretion traits such as plantain (Nguyen et al., 2022).

2.6.1. Perennial Ryegrass

Perennial ryegrass (*Lolium perenne* L.*)* is a commonly sown temperate grass species used in New Zealand pastures that is often used alongside white clover (Morris, 2013). Perennial ryegrass has a high feeding value compared to other grass species, is persistent in New Zealand conditions and has relatively high yields (Waghorn & Clark, 2004).

2.6.2. Narrow-leaved Plantain

Plantain (*Plantago lanceolata* L.) has been identified as a promising forage for reducing both nitrate leaching and N_2O emissions from ruminants such as sheep (O'Connell et al., 2016) and cattle (Cheng, McCormick, et al., 2017). Plantain can reduce nitrate leaching and N_2O emissions from ruminants because it exhibits a diluting effect on urine by

increasing urine volume, decreasing N concentration and reducing urinary excretion of N (Nguyen et al., 2022). This in turn can reduce the loss of N via GHG emissions and leaching. Inclusion of plantain into dairy cattle pastures has been reported to reduce N_2O emissions by 30-39% (Podolyan et al., 2020; Vi et al., 2023).

The exact mechanism by which plantain dilutes urine is unknown but it has been suggested that lower concentrations of DM may be responsible (Minneé et al., 2019). Typically, there is less DM in plantain than perennial ryegrass so to achieve a set DMI, a higher feed intake is required which can increase urine volume. However, in a study with sheep that balanced water intake, sheep fed plantain still excreted ~0.5L/d more than those fed perennial ryegrass (O'Connell et al., 2016). So, the increase in urine volume is not likely solely dependent on water intake.

Soluble protein (Minneé et al., 2019) and inorganic components of the diet such as sodium (McGusty, 2017b; O'Connell et al., 2016) have been identified as potential factors that have a diluting effect in urine. Rumen degradable protein (RDP) is positively related to increased urinary N excretion (Nennich et al., 2006) so decreasing soluble protein could theoretically reduce RDP and thus reduce urinary N excretion. Sodium on the other hand has been reported to have a small effect on urine volume in sheep (McGusty, 2017b).

2.6.3. Typical forage chemical composition.

The following table shows typical nutritional composition values of both perennial ryegrass and plantain alongside mixed swards containing these forages (Table 2.9). Generally, plantain has lower concentrations of crude protein (CP) and neutral detergent fibre (NDF) than ryegrass. The metabolizable energy (ME) values for ryegrass and plantain are similar but this is dependent on the time of year and season (Pain et al., 2015).

Plantain-clover mixes on the other hand, tend to have similar or greater CP and ME concentrations than perennial ryegrass-white clover swards and lower NDF concentrations; this likely contributes to the higher growth rates reported for animals grazing plantain-clover mixes (Table 2.9). Plantain-clover mixes can have higher ME and CP concentrations than ryegrass-white clover pasture because the lower density of

plantain allows a greater clover content (Cranston et al., 2015). Clovers such as red clover or white clover have high feeding values which can drive greater animal performance (Barry, 1998). For this reason, the quality of plantain-clover mixes is largely dependent on the amounts of clover and dead material in the sward (Cranston et al., 2018).

Table 2.9. Chemical composition of perennial ryegrass and plantain as individual forages and/or combined in a mixed sward.

Forage	ME (MJ ME/kg DM)	%DM		
		OMD	CP	NDF
Perennial Ryegrass				
(Pain et al., 2015) (Spring)	10.4		12.9	43
(Pain et al., 2015) (Summer)	9.7		21.7	44.6
(Sun et al., 2012)	9.7		21.7	44.6
Plantain				
(Pain et al., 2015) (Spring)	10		9.1	41.5
(Pain et al., 2015) (Summer)	10.7		17.7	34
(Cave et al., 2014)	10.4-11.5	71.5-79.7	12.6-24	26.1-41.3
Perennial Ryegrass-White Clover				
(Cranston et al., 2015)	8.9-10.6	64.0-72.0	9.0-25.0	36.0-62.0
(Somasiri et al., 2015b)	9.5-10.1	64.5-69.4	11.5-18.7	46.3-53.7
(Somasiri et al., 2016)	10.1-10.3	68.3-70.1	15.5-16.7	47.2-48.2
(Corner-Thomas et al., 2020)	8.3-10.1	51.9-63.4	7.3-24.9	44.6-66.8
Plantain-clover mix				
(Cranston et al., 2015)	9.8-11.4	68-83	9.0-25	24.0-49.0
(Somasiri et al., 2015b)	10.8	73.8-74.4	18.8-24.2	34.6-39.1
(Somasiri et al., 2016)	10.7-10.9	74.5-75	18.6	25.2
(Corner-Thomas et al., 2020)	8.3-11.2	51.6-70.2	9.0-25.3	26.2-44.1

2.6.4. Lamb growth rates when fed plantain or perennial ryegrass

Plantain alone has a variable effect on lamb growth rate (Table 2.10), likely the result of seasonal changes in its nutritional content and palatability (Pain et al., 2015). It is more beneficial for farmers to incorporate plantain into mixed herb-clover swards because lamb growth rates both pre- and post-weaning tend to be higher when fed herb-clover mixes rather than perennial ryegrass-white clover or plantain alone (Table 2.10; Table 2.11). The inclusion of plantain at 30% of pasture is believed to be sufficient to reduce urinary N excretion from dairy cattle by about 22% (Nguyen et al., 2022), so mixed swards

containing plantain therefore could be sufficient for a decrease in urinary N excretion to be observed while also promoting greater growth rates. However, over the first two years of one study, no difference in nitrate leaching was found between sheep-grazed pastures of perennial ryegrass-white clover or plantain-white clover, but all leaching levels were relatively small (0.37-9.68 kg N/ha/year) (Maheswaran, 2023). In the third year of this study the nitrate leaching from the plantain-clover pasture was greater as it had to be resown and sprayed because it didn't persist well.

Table 2.10. Post-Weaning Growth Rates of Lambs fed Perennial Ryegrass-white clover pasture, a plantain-based sward or plantain.

	Growth Rate (g/day)		
Reference	Perennial Ryegrass/ White Clover	Plantain/clovers/ chicory mix	Plantain
(Kemp et al., 2013)	231-237.5	279.5-285	121-219
(Somasiri, 2014)	321-322	360-367	
(Somasiri et al., 2015a)	190-244	262-329	
(Somasiri et al., 2015b)	120-169	226-231	
(Golding et al., 2011)	56-119	246-247	
(Parker et al., 2008)	93	192	
(Somasiri, 2014)	160-170	208-255	
(Kidane et al., 2014)	189		
(Scales et al., 1995)	134-182		
(Fraser & Rowarth, 1996)	98-136		
Average	176.2	268.3	170

Table 2.11. Pre-weaning Growth Rates of lambs assigned to different forage treatments

	Growth rate (g/d)	
Reference	Perennial Ryegrass/ White Clover	Plantain/clovers/chicory mix
(Hutton et al., 2011)	188	234
(Corner-Thomas et al., 2014)*	248-333	301-376
(Corner-Thomas et al., 2018)*	371-395	402-412
(Corner-Thomas et al., 2020)	199-214	233-276
(Kenyon et al., 2010)	274	256
(Kidane et al., 2010)	161	
Average (Single lambs)	336.8	372.8
Average (Twin lambs)	207.2	249.8

*Growth rates from twin lambs, otherwise lambs are singles.

2.7. Forage digestibility

Digestibility refers to extent to which ingested feeds are broken down and absorbed by an animal. Forage digestibility is an important factor in the feeding value of a forage and the interaction between chemical composition, forage intake and digestion by the animal (Waghorn & Clark, 2004). The feeding value of a forage can be referred to as the animal response to the consumption of a forage in terms of production, so the effect a forage has on traits including growth, growth efficiency and feed conversion ratio (FCR). Higher digestibility values are correlated with lower FCR values because less feed must be consumed to meet the requirements for a set amount of weight gain. Digestibility of forages is also used in calculations of how much to feed an animal or how many animals can be fed to their requirements with a fixed amount of feed.

When looking at the ranges of estimated organic matter digestibility (OMD) values reported for ruminants, similar values have been obtained for perennial ryegrass and plantain (Table 2.12). These values vary greatly, often in response to but not limited to forage composition, season and forage maturity, feed intake and animal species.

Table 2.12. Estimated DM and OM digestibility of forages for ruminants. (Dry Matter Digestibility, DMD; Organic Matter Digestibility, OMD).

Forage	Season	DMD (%)	Effective Degradability (%)[2]	OMD (%)	Source
		Grasses			
Perennial Ryegrass	Spring	75.5-77.9		78.4-81.5	(Garry et al., 2021)
Perennial Ryegrass	Summer	77-78		78.5-80.2	(Garry et al., 2021)
Perennial Ryegrass[1]	-		74	77.3	(Burke et al., 2000)
Perennial Ryegrass	Spring-Summer			72.1-83	(Jančík et al., 2011)
Perennial Ryegrass	-	81.4			(Ulyatt et al., 2005)
Perennial Ryegrass	-	78.6-83.5			(Della Rosa et al., 2022)
		Legumes			
Red Clover	-	75.6			(Waghorn et al., 2002)
Red Clover[1]	-		65	85	(Burke et al., 2000)
White Clover[1]	-		81	82.1	(Burke et al., 2000)
		Herbs			
Plantain[1]	-		81	85	(Burke et al., 2000)
Plantain	Autumn			74	(Navarrete et al., 2016)
Plantain	Autumn			71.5-79.7	(Cave et al., 2014)
Plantain	-	57.3-75.7			(Della Rosa et al., 2022)
Chicory	Autumn			75	(Navarrete et al., 2016)
Chicory	-	79.3			(Waghorn et al., 2002)
Chicory[1]	-		84	83.9	(Burke et al., 2000)
		Mixed Swards			
Ryegrass/White Clover	-	74			(Waghorn et al., 2002)
Ryegrass/White Clover	Winter-Spring (Jul-Sep)	82			(Ulyatt et al., 2002)
Ryegrass/White Clover	Spring-Summer (Nov-Mar)	73.35			(Ulyatt et al., 2002)
Herb-clover	Early Spring			84.1	(Cave et al., 2014)
Herb-clover	Late Spring			84.3	(Cave et al., 2014)
Herb-clover	Summer			78.1	(Cave et al., 2014)
Herb-clover	Autumn			81.2	(Cave et al., 2014)

[1] – Denotes results from an *in-vitro* study that focused on digestibility of these forages in dairy cattle, OMD results from this study were predicted using Near Infrared Spectrometry.
[2]- Effective degradability from *in sacco* incubation trial

2.7.1. Effect of forage chemical composition on digestibility.

Forage chemical composition can affect forage digestibility in a number of ways; high concentrations of NDF are associated with lower DM digestibility values whilst high levels of CP and readily fermentable carbohydrates such as sugar and starch are positively linked to DM digestibility. Neutral detergent fibre is comprised of cellulose,

hemicellulose and lignin which cannot be digested by animal enzymes. Microbes in the rumen can digest cellulose, hemicellulose and lignin (to a limited extent) but this occurs at a much slower rate than digestion of starch, protein and fats (Moore & Jung, 2001). The slower rate of digestion of NDF means that the amount of NDF in a forage is usually negatively related to DM digestibility. This is shown by decreasing DM digestibility of alfalfa fed to sheep when the amount of fibre in the diet was increased (Robles et al., 1981).

Forage chemical composition is dynamic and changes throughout the year. These changes are brought about by differences in rainfall and temperature associated with each season but also the growth patterns of forage species. In general, forages in a vegetative state have lower concentrations of NDF and higher concentrations of CP and water-soluble carbohydrates. More mature, reproductive forages on the other hand usually have higher NDF concentrations and are less digestible (Fonnesbeck et al., 1981).

For perennial ryegrass pastures, summer corresponds with when the forage is in its reproductive stage so NDF concentrations tend to be greater (Machado et al., 2005), whereas in early spring, NDF concentrations are lower and there is a greater amount of protein. Plantain has a different growth pattern than perennial ryegrass with greater growth rates in late summer and smaller pasture growth rates over winter (Somasiri et al., 2015b, 2016) Not only does plantain have a different pattern of growth than perennial ryegrass with a greater proportion of growth happening over summer, but NDF concentrations in plantain can be lower in summer as well (Pain et al., 2015). Plantain is also more tolerant of drought conditions (Stewart, 1996). Dry conditions can alter forage chemical composition, restrict pasture growth, and increase the proportions of dead matter and NDF in a sward. As plantain is more tolerant of dry conditions than perennial ryegrass, it is less likely for NDF concentrations and dead matter to be increased in response to drought. Dry matter digestibility is negatively correlated with NDF, so this means plantain is less likely to become less digestible in response to drought conditions.

It is important to note that not all NDF is equally digestible with variation both within and between forage species. A major factor of the digestibility of NDF is the content of lignin which is degraded slower than cellulose and hemicellulose. The structure and type of lignin found in and made by plants is believed to be chemically controlled and varies

with plant species, cell type and biochemical activity (Moore & Jung, 2001). content of lignin and its chemical linkages in forages has been found to explain a large proportion of NDF digestibility in forages fed to ruminants however this varies by forage (Raffrenato et al., 2017).

Plantain typically has lower concentrations of NDF than perennial ryegrass (Table 2.9) however in one study that fed plantain and perennial ryegrass to dairy cattle, it was found that plantain had a lower DM digestibility despite having a lower NDF concentration. Digestibility of NDF in this study was much lower for plantain which had greater amounts of indigestible NDF and lignin (Della Rosa et al., 2022). Similarly, sheep fed 25% plantain and 75% perennial ryegrass had a lower NDF digestibility than sheep fed perennial ryegrass alone, though DM digestibility was not significantly altered (Woodmartin et al., 2024).

2.7.2. Feed intake

Feed intake is a complex trait controlled by multiple mechanisms to ensure animals consume enough feed to meet their nutritional requirements. Feed intake is intrinsically related to forage digestibility. Both feed intake and digestibility affect and are affected by traits of digestion kinetics including rumen retention time. Rumen retention time refers to the amount of time ingested feed spends in the rumen which is controlled by the passage rate of feed in the rumen. The feed passage rate through the rumen is in turn controlled by the amount of feed in the rumen and the rate feed is broken down to small enough particles to leave the reticulo-omasal orifice (Okine et al., 1998).

When looking at varying feed intakes of a set forage (same quality and species), feed intake and rumen retention time are inversely related (Thornton & Minson, 1972). This means lower feed intakes of a forage are associated with feed being retained for longer in the rumen which can enhance forage digestibility (Dias et al., 2011). The reverse is also true that greater feed intakes can reduce forage digestibility (Long et al., 2004). Longer rumen retention times can improve digestibility because digesta spends longer in the rumen where it can be digested by microbes. At lower feed intakes, chewing efficiency can also be improved which means feed is physically broken down faster which can increase the rate of chemical digestion in the rumen (Dias et al., 2011). Varying feed

intakes of different forages (different in quality and/or species) however do not always show this negative relationship between feed intake and forage digestibility because more digestible forages require less time to be digested in the rumen and move through the rumen faster which can increase feed intake. This effect of forage digestibility on feed intake is limited by both gastrointestinal tract capacity and the nutritional requirements of the animal (Waldo, 1986).

Plantain typically has a lower dry matter content than ryegrass (Minneé et al., 2019), this can promote higher feed intakes to reach the same DM intake which was observed in the first experiment of a study with dairy cattle by Della Rosa et al. (2022). So, it is possible that digestibility of plantain might be lowered in part due to feed intake being greater.

2.7.3. Animal species

The species of animal used in digestibility trials can influence the results produced. For example, in an *in-vitro* digestibility trial comparing sheep and buffalo ruminal fluid, gas production was much faster when using the sheep ruminal fluid (Calabrò et al., 2005). This suggests that there can be differences in the efficacy of ruminal fluid from different ruminant species in fermenting feeds.

When it comes to the effect of animal species on forage digestibility with a focus on sheep perhaps the most important comparison is between that of sheep and cattle. In *in vivo* studies, sheep are often used as models for cattle because they are much smaller and cheaper making it easier to have larger scale experiments (Garry et al., 2021). On the other hand, *in-vitro* studies often use ruminal fluid from cattle in place of sheep because more ruminal fluid can be collected from one fistulated cow than one sheep and other practical considerations.

Fortunately, it appears that for moderate to high quality forages there is not much difference in forage digestibility when measured in sheep or cattle (Soto-Navarro et al., 2014). This is further supported by Garry et al. (2021) as the digestibility values reported from sheep and dairy cattle fed perennial ryegrass in spring were similar. However, with lower quality forages differences in digestibility can arise. When feeding lovegrass hay (*Eragrostis curvula)*, a low quality forage with high NDF and low CP concentrations to

steers and wethers, organic matter digestibility was lower for the wethers by about 15.6%, digestibility of NDF and ADF were also lower for the wethers by approximately 20% (Soto-Navarro et al., 2014). In another study, dairy cattle had lower DM and OM digestibility values than sheep fed summer perennial ryegrass (Garry et al., 2021). These differences in digestibility highlight how ruminant species can but not always affect the results of digestibility trials.

2.8. Research Opportunity

The pattern of creatinine excretion from dairy cattle has been well studied and used to estimate urinary outputs with varying degrees of success. This is not the case with sheep and relatively little is known about how factors such as the time of day affect creatinine excretion. There have also been variable results when comparing creatinine excretion of sheep fed different diets. This research will investigate whether there is a consistent pattern to creatinine excretion in sheep while also identifying if there are any diet-dependent differences in creatinine excretion that come from feeding perennial ryegrass or plantain to lambs. This will allow for assessment of how suitable or viable it is to predict urinary output traits such as volume using creatinine in sheep. It is important that species-specific studies are undertaken because creatinine excretion rates differ significantly between cattle and sheep and so it cannot be assumed that the findings from studies in cattle can be applied to sheep. This will be of use to researchers in the sheep industry looking at quantifying urinary output by either supporting the use of creatinine to estimate urinary outputs and potentially identifying the most appropriate time to sample urine.

Similarly, there is a gap in recent knowledge regarding the digestibility of some forages fed to sheep such as plantain which will be addressed by this research. Several of the digestibility studies using sheep in New Zealand were conducted over a decade ago; this presents a challenge to application of these results because both forage cultivars and sheep have undergone change due to selective breeding. The digestibility of forages is important as it affects the quantity of nutrients available to the animal and how much has to be consumed to reach a set nutrient intake. Forage digestibility is also used when considering methane emissions.

2.9. Objectives

For this experiment there are two research objectives. The first is to investigate the pattern of creatinine excretion from sheep fed perennial ryegrass or plantain at different times of day. This objective is focused on identifying if it is possible to accurately predict urinary output from creatinine in sheep and determine whether or not there is a time period over which spot samples of creatinine are most likely to provide accurate results. It is expected that feeding plantain or ryegrass will not affect the total amount of creatinine excreted, but will likely significantly alter the concentration of creatinine in urine due to the diluting effect of plantain on urine.

The second objective is to measure the *in vivo* digestibility of the two forages used with sheep in New Zealand. It is hypothesised that plantain will have a lower DM digestibility *in vivo.* Based on previous studies, the lower NDF digestibility of plantain may make it less digestible than perennial ryegrass.

3 Materials and Methods

This research was carried out at Massey University, Palmerston North in New Zealand with the approval of Massey University Animal Ethics (AEC 23/60). The experiment was undertaken during February (late summer) of the year 2024.

*3.*1. Experimental Design

Twelve castrated Romney male lambs, approximately 6-months of age were used in a randomised complete block experimental design for eight days. The lambs weighed on average 30.6 kg and ranged between 23.5 and 38 kg. Before the experiment these lambs were raised in the same mob and grazed predominantly perennial ryegrass-based pasture. Some of the lambs used had previously been used in another *in vivo* digestibility trial about a month earlier. Prior to the experimental period, the lambs were weighed and then blocked into two groups by liveweight before being allocated to one of two dietary treatments: perennial ryegrass (*Lolium perenne* L.) (n=6) and plantain (*Plantago lanceolata* L.) (n=6). This was done to ensure the two treatment groups had similar average liveweights (30.5 and 30.8 kg).

For the duration of the experiment lambs were kept indoors. The first 3 days of the experiment was the adaptation period during which the sheep were placed in individual pens (1.94 m^2) and given free access to their allocated feed. On the morning of day 4, the lambs were all moved to metabolism crates (0.465 m^2) where they remained for a 5-day collection period. The metabolism crates were arranged so that all animals could easily see other lambs.

During the collection phase of the experiment feed offered, feed refused, urine and faeces were all collected and weighed. The metabolism crates were fitted with collecting funnels for urine collection. To reduce faecal contamination of the urine, lambs were fitted with harnesses and faecal collection bags. Free access to clean water was provided to all the lambs for the duration of the trial.

3.2. Dietary Treatments

The dietary treatments for this experiment were Perennial Ryegrass (*Lolium perenne* L. cv. Maxsyn) and Plantain (*Plantago lanceolata* L. cv. Agri Tonic). The forages used were

sown as monocultures in spring of 2023 at a nearby site. Forage diets were cut fresh, daily, in the morning using a sickle bar mower and stored in a cool location to prevent wilting until required. Feed was allocated in excess of voluntary feed intake for each sheep and sheep were fed twice daily at 9 am and 3 pm. However, due to the forages bulk the feeders were not big enough to hold the feed provided at each feed, so the level of feed in each feeder had to be frequently checked to ensure there was always feed available, and delivery of the morning and afternoon feeds was often spread over multiple hours to ensure lambs had *ad libitum* access to feed.

Table 3.1. Chemical composition of plantain and perennial ryegrass offered during the experiment measured using wet chemistry.

Item[1]	Plantain	Ryegrass	P-value
DM (%)	21.2 ± 1.0	29.6 ± 3.0	0.115
GE (kJ/g)	15.8 ± 0.1	15.9 ± 0.1	0.291
CP	9.3 ± 0.1	12.5 ± 0.3	0.008
Ash	10.1 ± 0.03	12.7 ± 0.7	0.077
Fat	2.3 ± 0.1	3.4 ± 0.5	0.162
NDF	39.5 ± 2.4	51.3 ± 0.6	0.042
ADF	30.4 ± 2.5	27.8 ± 0.1	0.413
Lignin	8.5 ± 0.7	3.5 ± 0.1	0.02

[1] Expressed in % DM (dry matter) unless otherwise stated. CP=Crude Protein, GE=Gross Energy, NDF=Neutral Detergent Fibre, ADF=Acid Detergent Fibre.
[2]Values are means ± standard errors (SE).

Feed samples from day 1 and 2, and days 4 and 5 of the collection phase were pooled and analysed for chemical composition. The average of these values and standard error is shown in Table 3.1. Notably the Dry Matter (DM) content of both forages was relatively high due to limited rainfall both prior to and during the experiment.

3.3. Measurements

The lambs were weighed using a weigh scale on day 1 and day 8. Their average daily gain was calculated as the difference between these weights divided by 8 days representing the length of the experiment.

During the collection phase, the total collection method was implemented which included weighing in all feed and water offered, all feed and water-refused, and all faeces were removed and weighed daily. Feed refusals included feed remaining in the feeders, on the floor of the crate and the floor near the crate that had been dropped by each

animal. Water refusal was measured as the weight of water left in the water bowls, spillage and evaporation were not accounted for. Faeces were collected in plastic bags that lined the inside of the faecal collection bags. When the faeces were collected each day, this plastic lining was replaced with a new one.

Samples were taken of the feed refused by all sheep in each treatment group daily and then pooled into one sample of feed refused per treatment. Daily samples were taken from each sheep's faeces. Samples of each feed offered (a minimum of 100g) were taken in the morning. The feed samples from day 1 and 2 were pooled and the samples from day 4 and 5 were pooled. Both samples were analysed for chemical composition. All the feed offered, feed refusals and faecal samples were stored in a freezer in resealable plastic bags.

Urine was collected 5 times per day at 6 am, 9 am, 12 pm, 3 pm and 6 pm; except for the first day of the collection phase (day four of the experiment) when urine weight and samples were only taken at 6pm because there was very limited urine output. Each of the metabolism crates had a collecting funnel which directed the animal urine into a bucket. At each sampling time, the urine was weighed by subtracting the weight of the bucket from the total weight of the urine and bucket. At each sampling time, two 100mL samples were collected for each animal (one non-acidified and one acidified with 98% sulphuric acid) where possible.

3.4. Chemical Analysis

Prior to chemical analysis of the samples, the samples of feed offered, feed refusals and faeces were weighed and then freeze-dried. Post-freeze-drying weights were recorded and then the samples were ground. Samples were freeze-dried and ground at the Massey University Poultry Unit. Then all samples were sent to the Massey University Nutrition Laboratory for chemical analysis.

The feed samples and faecal samples were analysed for gross energy (GE), moisture content, and nitrogen (N), ash, fat, neutral detergent fibre (NDF), acid detergent fibre (ADF) and lignin, on a DM basis. Organic matter was calculated as the ash in a forage subtracted from the DM fraction. The acidified urine samples were tested for N content

whilst the non-acidified urine samples were tested for gross energy and creatinine, urea and allantoin concentrations. The methodologies are given below.

To determine moisture content feed and faecal samples were further dried according to AOAC methods 925.10 and 930.15. The moisture content from these samples were then used to determine the DM content of the samples prior to freeze-drying. To calculate DM the following equation was applied where post-freeze drying weight was the sample weight after freeze drying and moisture content solely refers to the moisture content returned by the lab for the freeze-dried sample.

$$Dry\ Matter\ (\%) = \frac{Post.freeze\ drying\ wt - Po\quad .freeze\ drying\ wt \times moisture\ content}{Initial\ Sample\ wt} \times 100\%$$

Where, wt = weight (kg).

Total N content of samples were determined using the Dumas method (AOAC 968.06). Where appropriate, N values were multiplied by 6.25 to estimate crude protein which is based on the assumption that all N in these samples comes from protein and that N makes up 16% of protein.

Gross Energy (GE) in kJ/g was measured using a bomb calorimeter while ash content was found using a furnace at 550°C according to AOAC method 942.05. The NDF, ADF and lignin were all found using a Fibertec system that employed AOAC methods 2002.04 and 973.18. Crude Fat was found using hexanes extraction AOAC 2003.06.

Creatinine and allantoin concentrations of urine were found using colourimetric methods with picric acid used to determine creatinine concentration (Bartels et al., 1972; Henry et al., 1974). Urea concentration was found by urease kinetic UV assay (Bergmeyer, 1965; Tietz, 1976).

3.5. Statistical Analysis

During this study, one lamb needed to be euthanised for reasons unrelated to the treatment resulting in n=5 for the plantain treatment group. There was also a small number of sampling times (n=11) where no or only one sample was collected from an animal due to inadequate amounts of urine for sampling.

Apparent faecal digestibility coefficient values for DM, OM, GE, ash, fat, NDF, ADF, lignin and N were calculated from the data by dividing the nutrient intake minus the content of that nutrient excreted in faeces by the total nutrient intake on a daily basis. The digestible content of DM, GE, ash, fat, NDF, ADF, lignin and N per kg DM intake was calculated using the total content and digestibility coefficients.

Because of low feed intake and high faecal output, together with multiple calculated digestibility values from lamb numbers 267 and 289 being negative, data from these lambs was excluded from the digestibility dataset and from calculations that involved faecal excretion values including N intake and excretion and subsequently proportions of N retained and excreted in faeces or urine. This brought the number of lambs in the ryegrass group for digestibility calculations down from six to four. These abnormal values were likely stress induced as it was noted during the experiment that 289 appeared more stressed than the other sheep whilst in the metabolism crates and that the faeces were less structured and had a lower DM content (17.2% average for 289 compared with 30.8% average for all other sheep with standard deviation of 3.8%).

Metabolisable energy (ME) values for each animal over the whole experiment were calculated by subtracting the GE lost in faeces and urine from the GE intake. The GE lost as methane was then estimated by first using the following equation from Swainson et al. (2016) to calculate the methane production per sheep per day, and then multiplying the methane in kg by 55.65 MJ GE. The ME values were then corrected.

$$\ln(pCH_4) = 0.763 \times \ln(DMI) + 3.039$$

Where, CH_4 = methane (g/d); DMI = dry matter intake (kg DM/d).

For descriptive values such Average Daily Gain (ADG), liveweights, intake of feed and water which were not of focus, but important for the purpose of this study, averages from each animal were collected and analysed with single factor Analysis of Variance (ANOVA) in Microsoft Excel.

All other data analysis was carried out using the GLM procedure on the 9.4-bit version of SAS (SAS Institute Inc., Cary, NC, USA) with repeated measurements. Day was used as the repeated value. The significance level utilised was 0.05. Nutrient digestibility values

were analysed using a model that had forage treatment as a fixed effect. For the concentration and excretion of compounds in urine, forage treatment and time of day were used as fixed effects where applicable whereas day and animal ID which was nested in forage treatment were random effects.

The interaction between treatment and time was included in all analyses that took time into consideration to investigate whether any differences observed at different times of the day were affected by the forage given. For the analysis of the concentration of urine, the interaction between treatment and day was undertaken to examine if there were differences due to changes in the forages over the experiment because the forages were cut in the morning each day. The interaction between day and time was used for the hourly excretion rates of urine, creatinine, urea and nitrogen.

The creatinine concentrations from each animal at each sampling time were used to predict urine volume using published equations from (Jonker et al., 2021; Maheswaran et al., 2022; McGusty, 2017a). The predicted urine volumes were than compared to the measured daily urine volume using a scatterplot with predicted urine volume on the y-axis and observed urine volume on the x-axis, the proximity of the plotted values to the line y=x was used as an indicator of accuracy. The same process was then applied to the mean daily creatinine concentration of each sheep for days 2, 3, and 4 which was determined as the ratio of daily creatinine excretion in mmol to the volume of urine excreted.

4 Results

4.1. Feed intake, growth rates and faecal output of sheep over the experiment.

There was no difference in ADG, initial, and final liveweight between the two treatment groups ($p>0.05$) (Table 4.1). Both groups of lambs had mean ADGs of less than 250g/d. Similarly, the mean DMI was unaffected by forage treatment group ($p>0.05$). However, the plantain lambs had a larger mean feed intake (FI) and water intake ($p<0.05$) than the sheep allocated to ryegrass. Despite the greater FI, the plantain lambs had a lower ($p<0.0001$) mean faecal DM output. There was also no difference in faecal DM % between the lambs fed plantain or ryegrass ($p>0.05$).

Table 4.1. Mean ± SE of feed intake, water intake, growth rates, and faecal output of the sheep over the experiment by treatment. (average daily gain, ADG; liveweight, LW; feed intake (using the wet weight of feed), FI; dry matter intake, DMI; dry matter, DM).

	Plantain	Ryegrass	P-value
ADG (kg/d)	0.085 ± 0.096	0.227 ± 0.075	0.266
Initial LW (kg)	30.8 ± 1.67	30.5 ± 2.27	0.906
Final LW (kg)	31.5 ± 1.5	32.3 ± 2.03	0.775
Average FI (kg/d)	7.02 ± 0.33	4.95 ± 0.26	0.001
Average DMI (kg/d)	1.4 ± 0.07	1.26 ± 0.09	0.266
Average Water Intake (kg/d)	5.7 ± 0.27	4.1 ± 0.19	<0.001
Average Faecal DM output (kg/d)	0.27 ± 0.015	0.375 ± 0.01	<0.001
Faeces DM %	31.8 ± 1.1	27.654 ± 2.4	0.169

4.2. Urine concentration

Treatment and day of the experiment had significant effects on all of the analysed components of urine (Table 4.2). The time of day and animals was also largely influential with Gross Energy being the only component not affected ($p>0.05$). The interaction between treatment and day was always significant whilst the interaction between treatment and time of day was only significant for N, urea and GE concentrations. Overall, the concentrations of N, creatinine, urea, allantoin and GE were greater ($p<0.05$) for the ryegrass lambs than the plantain lambs.

Table 4.2. Least square means and standard errors of the concentration of nitrogen (N), creatinine, urea, allantoin and gross energy (GE) in urine

			p-values					
	Plantain	Ryegrass	Treatment	ID (Treatment)	Time	Day	Treatment *Time	Treatment *Day
N (%)	0.16 ± 0.14	0.75 ± 0.13	0.0131	<.0001	<.0001	0.0006	<.0001	<.0001
Creatinine (µmol/L)	1324 ± 431	2857 ± 393	0.0275	<.0001	<.0001	0.0001	0.1992	0.0055
Urea (mmol/L)	26.6 ± 39.9	193.8 ± 36.4	0.0129	<.0001	<.0001	<.0001	<.0001	<.0001
Allantoin (mg/L)	410 ± 141	1052 ± 129	0.0083	<.0001	0.0056	0.0004	0.3443	0.0159
GE (kJ/g)	2.6 ± 0.23	3.8 ± 0.21	0.0036	0.3152	0.0548	<.0001	0.0366	0.0002

Table 4.3. Concentration of creatinine, nitrogen (N) and urea in the urine of sheep from the experiment by treatment and time of day. Means and SE.

	Creatinine (µmol/L)		N (%)		Urea (mmol/L)	
Time	Plantain	Ryegrass	Plantain	Ryegrass	Plantain	Ryegrass
6pm-6am	1325^{a} ± 173	2674^{cd} ± 157	0.14^{a} ± 0.04	0.61^{c} ± 0.03	26^{a} ± 10.8	141.5^{d} ± 9.8
6am-9am	1905^{b} ± 179	3092^{c} ± 167	0.20^{a} ± 0.04	0.67^{c} ± 0.04	40.1^{a} ± 11.2	192.1^{c} ± 10.4
9am-12pm	1310^{a} ± 184	3201^{c} ± 164	0.18^{a} ± 0.04	0.92^{b} ± 0.04	28.4^{a} ± 11.5	239.2^{b} ± 10.2
12pm-3pm	1125^{a} ± 179	2829^{cd} ± 164	0.14^{a} ± 0.04	0.86^{b} ± 0.04	17.1^{a} ± 11.2	220.8^{b} ± 10.2
3pm-6pm	958^{a} ± 179	2489^{d} ± 164	0.14^{a} ± 0.04	0.69^{c} ± 0.04	21.5^{a} ± 11.2	175.3^{c} ± 10.2

^{a,b,c,d,} Different superscripts denote statistical differences ($p<0.05$) between rows and columns within each measured value determined using LSD test.

Creatinine concentration from both forage groups was affected by time ($p<0.001$), though the interaction between treatment and time was insignificant ($p>0.05$) (Table 4.2). The plantain lambs highest mean creatinine concentration was for the 6am-9am interval (Table 4.3). All other intervals were similar for the plantain fed lambs. For the ryegrass lambs, creatinine concentration from the morning time periods (6am-9am and 9am-12pm) were greater than the 3pm-6pm but not the 6pm-6am or 12pm-3pm periods. On a daily basis, the lambs fed plantain had an average creatinine concentration of 1168 µmol/L whilst the lambs fed ryegrass averaged 2923 µmol/L.

Both the effect of time and the interaction between treatment and time were significant on the concentration of N in urine (Table 4.2), however when looking at the effect of time within forages it is notable that there was no effect of time on the N concentration in urine from the plantain group (Table 4.3). For the ryegrass lambs, the highest urine N concentrations were observed from the 9am-12pm and 12pm-3pm time periods with means of 0.924 and 0.858% respectively.

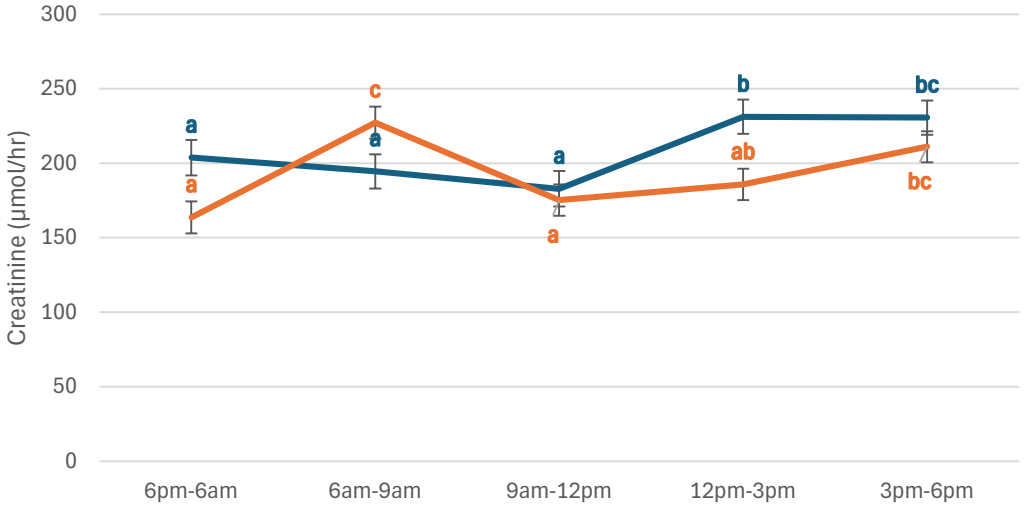
Urea concentration from the urine of the plantain-fed lambs was unaffected by time whilst time affected urine urea concentration from the ryegrass-fed lambs. For the ryegrass lambs, similarly as to urine N concentration, the highest urea concentrations were observed for the 9am-12pm and 12pm-3pm intervals. Though concentration of urea from the 6pm-6am period was significantly lower than all other values as well.

4.3. Urinary excretion of creatinine, N, and urea by treatment and time of day.

On average, lambs fed plantain excreted 209 ± 13.4 µmol of creatinine per hour which was not different ($p>0.05$) to the mean amount of creatinine excreted per hour from the ryegrass lambs of 193 ± 12.2 µmol. Time of day and the interaction between time and treatment both had significant effects on creatinine excreted per hour (Figure 4.1). Peak creatinine excretion occurred in the 6am-9am period for the lambs fed ryegrass whilst lambs fed plantain excreted more creatinine from 12pm-3pm.

On a mmol of creatinine excreted daily per kg $LW^{0.75}$ basis, there was also no significant difference between the two treatment groups ($p>0.05$). The plantain group had a mean of 0.343 ± 0.013 while the ryegrass group had a mean of 0.309 ± 0.012 mmol/kg $LW^{0.75}$.

Total urine volume and the effect of time of day on urine volume was more pronounced for the plantain lambs (Figure 4.1). On average, the lambs fed either forage excreted more urine per hour during the 3pm-6pm period. The plantain lambs had a mean rate of 0.28 ± 0.01 L/hr whilst the ryegrass groups mean rate was 0.096 ± 0.01 L/hr.



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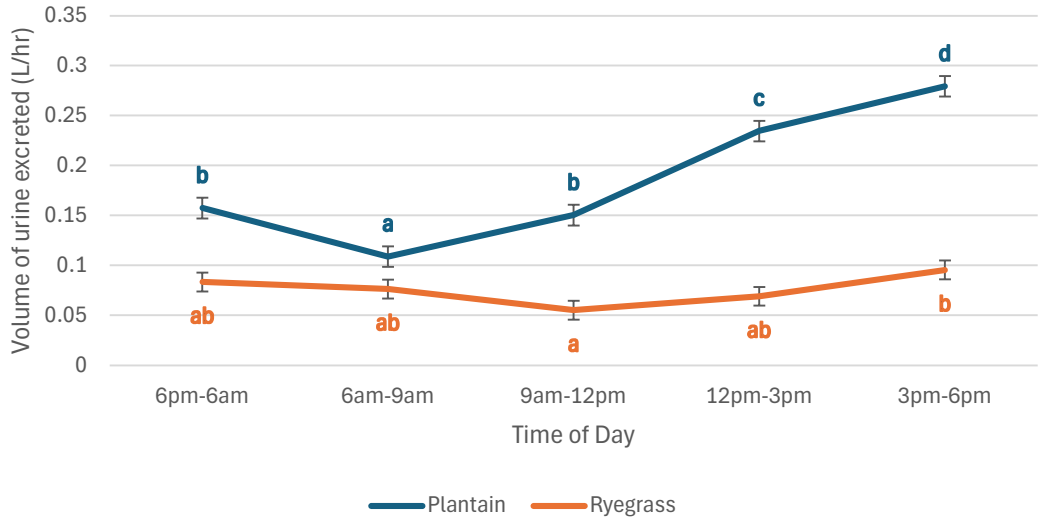


B

Figure 4.1. Mean hourly excretion rate of creatinine (µmol) (graph A) and urine volume (L) (graph B) from sheep by treatment and time of day. Error bars represent the standard error. Different superscripts indicate p-values of less than 0.05 determined from LSD test within forage treatment groups.

The interactions between day and time and treatment and time influenced ($p<0.05$) both hourly urinary N and urea excretion rates. Similarly, animal, time of day and treatment

all exhibited significant effects (Figure 4.2). For all time periods, the mean amount of nitrogen and urea excreted by the plantain group was lower ($p<0.05$) than that of the ryegrass lambs. The lowest means for nitrogen excretion from both forage groups came from the 6pm-6am period. Greater mean nitrogen excretion values were observed from later time periods and appeared to increase throughout the day. The daily mean rate of urinary N excretion for each group was 0.247 ± 0.027g N per hour for the plantain lambs and 0.49 ± 0.025gN per hour for the ryegrass lambs.

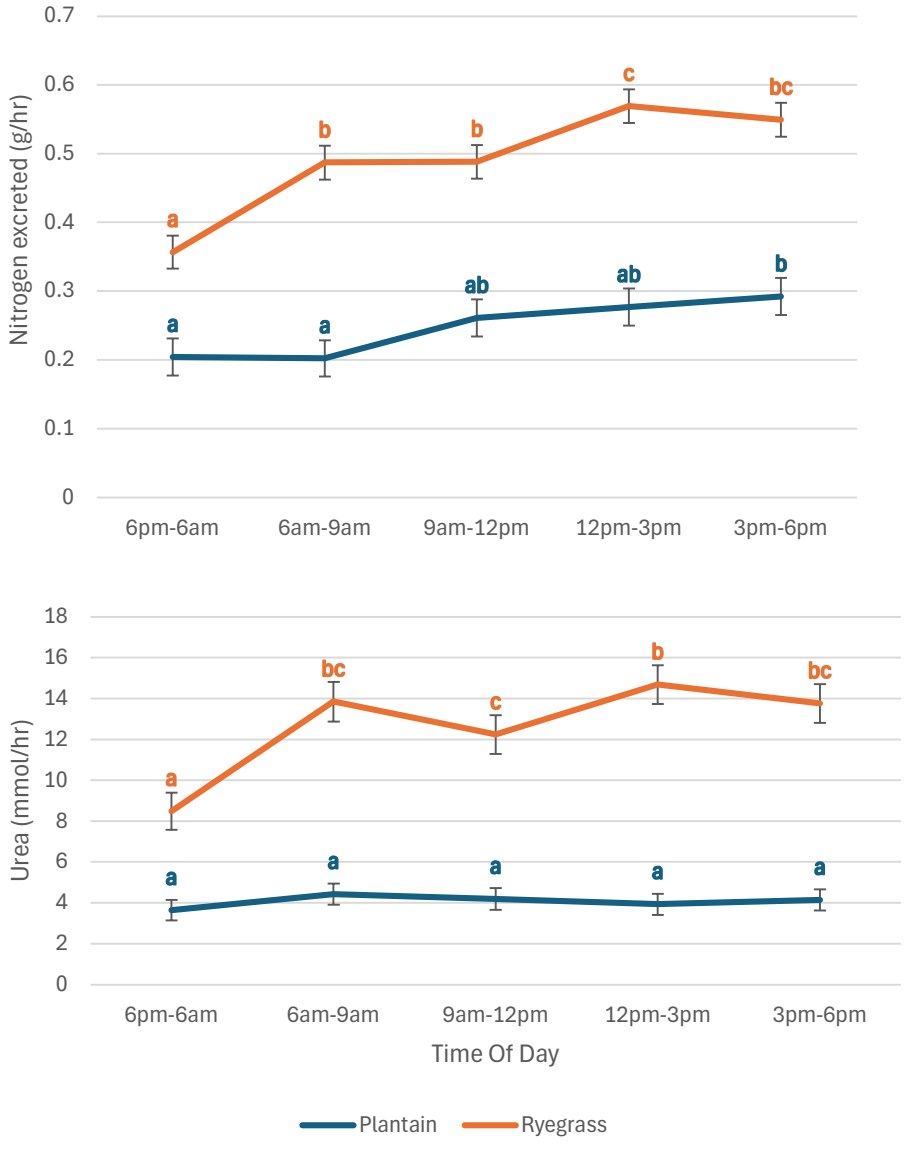


Figure 4.2. Mean hourly urinary excretion rate of nitrogen (graph A) (g) and urea (graph B) (mmol) from sheep by treatment and time of day. Error bars represent SE. Different superscripts indicate p-values of less than 0.05 determined from LSD test within forage treatment groups.

On average urea was excreted at a rate of 3.87± 0.63 mmol per hour for the plantain lambs and 12.30 ± 5.73 mmol per hour for the ryegrass group. Mean urea excretion rate from the plantain lambs was unaffected by time of day whilst time exhibited significant effects on the ryegrass lambs. The ryegrass lambs tended to excrete a greater amount of urea in the afternoon periods with the highest mean urea excretion rate being 14.7 mmol/hr for the 12pm-3pm period, though this was not significantly greater than the means for the 6am-9am and 3pm-6pm periods. The lowest mean for the ryegrass lambs was 8.5 mmol/hr for the 6pm-6am period.

4.4. Relationship between urine volume and water intake.

Daily water intake and urine volume in the experiment had a strong positive relationship with a correlation coefficient of 0.766 and an R-squared value of 0.5865 (Figure 4.3).

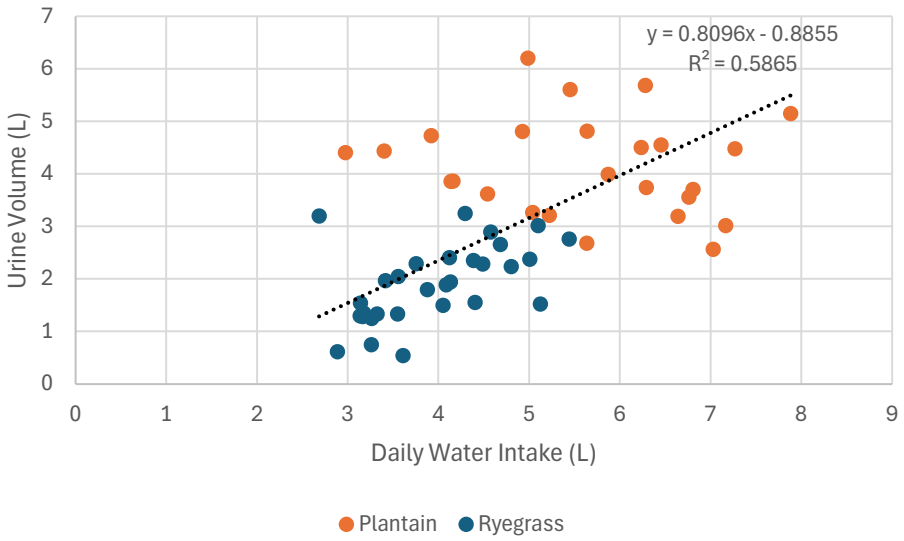


Figure 4.3. Graph of daily urine volume (L) by daily water intake (from both feed and water) (L) of all lambs from the experiment.

4.5. Relationship between creatinine concentration and urine volume excreted hourly.

Creatinine concentration had a strong negative relationship with urine volume, with a correlation coefficient of -0.727 and an R-squared value of 0.5859 (Figure 4.4).

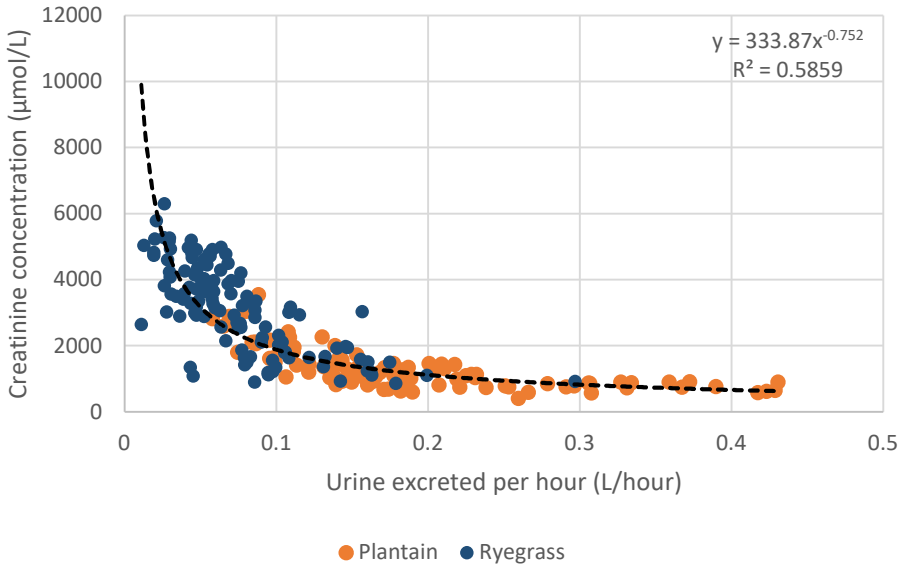
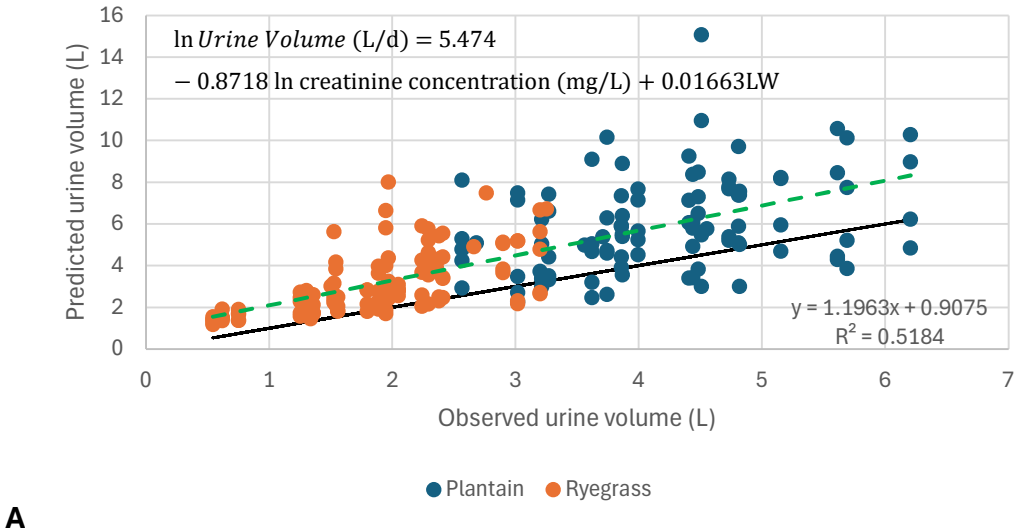


Figure 4.4. Relationship between creatinine concentration and the volume of urine excreted per hour for each sample time.

4.6. Applying Established Equations for Predicting Urine Volume to Study Data.

The measured daily urine volumes have been compared with predicted urine volumes using published equations (Jonker et al., 2021; Maheswaran et al., 2022; McGusty, 2017a). These are displayed in figures 4.5 and 4.6. In Figure 4.5 the creatinine concentrations measured at all sample times from each lamb were used. Figure 4.6 only used the mean daily creatinine concentration which was calculated as the ratio of the total amount of creatinine to urine volume excreted.

The equation by Maheswaran et al. (2022) adapted from data from McGusty (2017b) shown in figure 4.5B explained a more of the variation than the other two equations (figure 4.5A and 4.5C). In our experiment, the measured daily urine volumes varied between 0.5 to 6.2 L per day whilst the predicted daily urine volumes for each equation are 1.17 to 15.1 L/d (figure 4.5A), -1.7 to 5.8 L/d (figure 4.5B), and 0.61 to 12.1 L/d (figure 4.5C).



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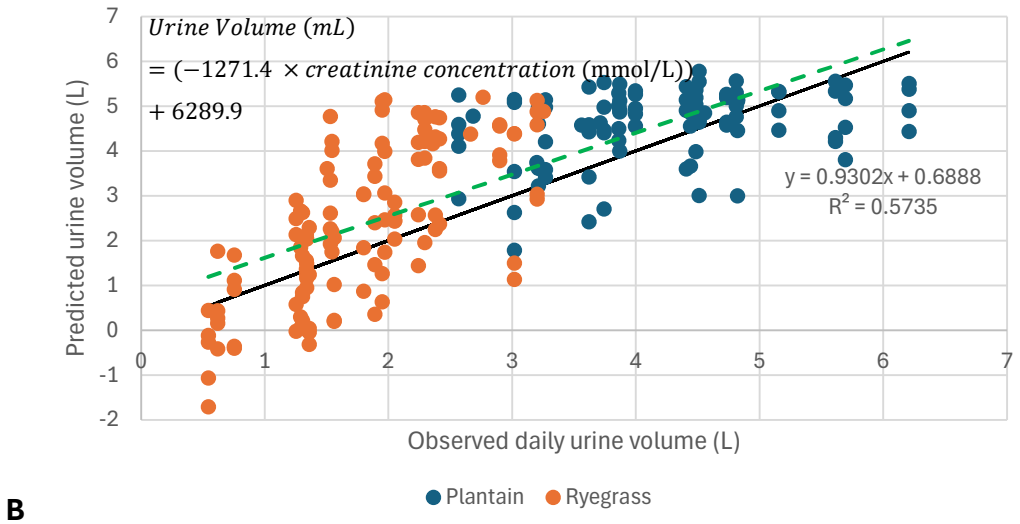

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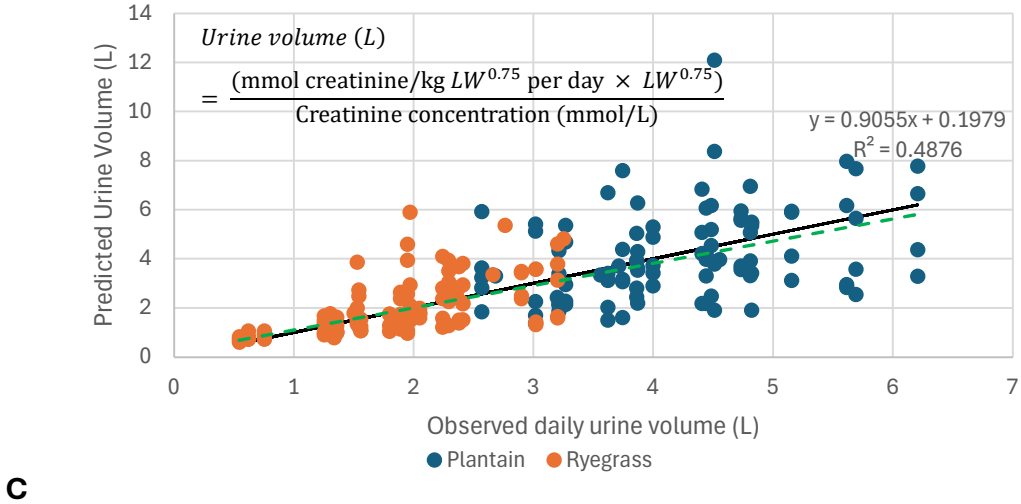

C

Figure 4.5. Daily urine volume (L) predicted using creatinine concentration against measured daily urine volume from sheep by forage treatment. (A) Uses equation from Jonker et al, (2021). (B) Uses equation from Maheswaran et al, (2022) created from data from McGusty (2017). (C) Uses an equation adapted from Maheswaran et al (2022). This equation also used the average daily creatinine excretion rate from days 2-4 of the

experiment when applied to our data. The solid black line represents the observed daily urine volume, and the dashed green line represents the trend in predicted urine volume for each equation.

The mean daily creatinine concentration was calculated for each of the lambs for days 2,3 and 4 and then used to estimate urine volume with the three equations. Figure 4.6 shows the relationship between the predicted and observed urine volume. When using the daily mean creatinine concentration, the predicted urine volume was less variable relative to the urine volumes predicted when using the creatinine concentrations measured from different periods. The ranges of urine volumes predicted using all three equations was -0.73 L/d to 10.5 L/d. Higher urine volumes were more likely to be overestimated.

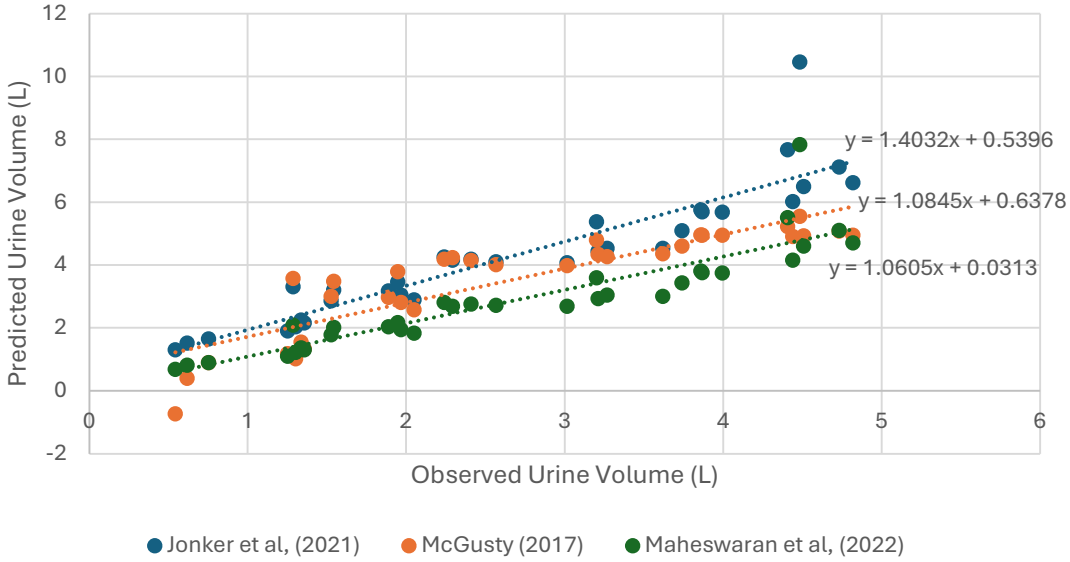

Figure 4.6. Predicted daily urine volume from all 3 equations using observed daily urine volume and mean daily creatinine concentration from days 2, 3, and 4 of experiment. Trendline intercepts were set at zero.

4.7. Nutrient Digestibility of forages

Day and individual animal (ID(Treatment)) both exhibited significant effects on all the apparent faecal digestibility coefficients described in Table 4.4. In this experiment, forage treatment did not have an effect ($p>0.05$) on the apparent faecal digestibility coefficients of GE, DM, ash, fat, NDF, ADF or N (Table 4.4). However, lignin digestibility was greatly affected by forage treatment with plantain having a much greater mean of 76.63% ±

3.97% than the mean for ryegrass of 42.71% ± 4.44% ($p<0.001$). The metabolizable energy per kg DM was also greater for the ryegrass ($p<0.05$).

Table 4.4. Faecal digestibility coefficients of dry matter (DM), organic matter (OM), gross energy (GE), ash, fat, acid detergent fibre (ADF), neutral detergent fibre (NDF), lignin and N by forage treatment and metabolisable energy (ME) content (MJ ME /kg DM). (Lsmean ± SE). Data analysed using repeat measure analysis model with forage treatment, day and ID as variables using SAS 9.4-bit version.

	Plantain (n=5)	Ryegrass (n=4)	P-values			
			Treatment	Day	ID (Treatment)	Day* Treat
ME (MJ ME/kgDM)	7.46 ± 0.5	9.39 ± 0.6	0.0361	0.0007	0.0328	0.1752
GE (%)	81.6 ± 1.9	77.3 ± 2.2	0.2361	0.0019	<0.0001	0.1447
DMD (%)	81.2 ± 1.9	77.8 ± 2.2	0.3809	0.0007	0.0001	0.1032
OMD (%)	84.5 ± 1.7	80.2 ± 1.9	0.1794	0.0019	<0.0001	0.1315
Ash (%)	52.8 ± 3.6	62.2 ± 4.2	0.0917	0.002	0.0048	0.1825
Fat (%)	68.5 ± 2.5	65 ± 3	0.493	0.0013	0.0045	0.0227
NDF (%)	80.9 ± 1.9	77.4 ± 2.4	0.3751	0.0006	0.0002	0.1208
ADF (%)	81.1 ± 1.7	76.6 ± 1.9	0.1645	0.0002	0.0016	0.1127
Lignin (%)	76.6 ± 3.6	43.8 ± 4.2	0.0007	<0.0001	<0.0001	0.006
N (%)	67.5 ± 2.5	74 ± 2.9	0.0907	0.0022	0.0023	0.2128

Whilst most of the digestibility coefficients were unaffected ($p>0.05$) by treatment, the digestible content of ash, fat, ADF, lignin and N was altered ($p<0.05$) on a per kg DMI basis (Table 4.5). The ryegrass had greater amounts of digestible ash, fat and N than the plantain. Plantain on the other hand had greater ($p<0.05$) amounts of digestible lignin and ADF per kg DMI.

Table 4.5. Digestible content of the two forage diets used in the experiment. (lsmean ± SE). (On a g/kg DMI basis unless otherwise specified). (Dry matter, DM; digestible energy, DE; metabolisable energy, ME; acid detergent fibre, ADF; neutral detergent fibre, NDF; nitrogen, N). Data analysed using repeat measure analysis model with forage treatment, day and ID as variables using SAS 9.4-bit version.

	Plantain (n=5)	Ryegrass (n=4)	P-values			
			Treatment	Day	ID(Treat)	Day*Treat
DE (MJ/kg DMI)	14.1 ± 0.3	13.4 ± 0.4	0.197	0.0006	<0.0001	0.0869
Ash (g/kg DMI)	57.3 ± 4.6	83.6 ± 5.3	0.0071	0.0001	0.0016	0.0077
Fat (g/kg DMI)	15.5 ± 0.8	24.5 ± 0.9	0.0002	<0.0001	0.0048	<0.0001
NDF (g/kg DMI)	357.8 ± 9.4	362.5 ± 11	0.7591	0.0172	0.2993	0.2087
ADF (g/kg DMI)	258.4 ± 5.6	231.9 ± 6.6	0.0184	<0.0001	0.0149	0.0003
Lignin (g/kg DMI)	70.8 ± 1.5	16.3 ± 1.7	<0.0001	<0.0001	0.0201	<0.0001
N (g/kg DMI)	11.1 ± 0.5	16.3 ± 0.6	0.0003	0.0001	0.0003	0.0211

4.8. Nitrogen excretion

The interaction between day and treatment had an effect ($p<0.05$) on urine volume, urine N concentration and N intake but not on N excreted or the proportions of N excreted and retained ($p>0.05$) (Table 4.6). Day affected ($p<0.05$) all values shown in Table 4.6 except for urine volume. Animal ID nested in treatment affected ($p<0.05$) urine volume, urine N concentration and the daily amounts of N intake and N excreted but not N excretion as a proportion of intake or the partitioning of N between urine and faeces.

Feeding plantain increased ($p<0.001$) urine volume by about 2L with the ryegrass group averaging 1.8L and the plantain group averaging 3.8L per day (Table 4.6). The plantain lambs also had a much lower ($p<0.01$) mean concentration of N in the urine than the ryegrass lambs ($0.12 < 0.59$).

Both mean daily N intake and excretion were higher for the lambs fed ryegrass ($p<0.01$) than those fed plantain. The mean proportion of N intake excreted was not significantly different ($p>0.05$) between forages, however the ryegrass group on average excreted a greater proportion of N intake in urine ($p<0.01$). This greater proportion of N intake in urine also corresponds in a change in the proportions of excreted N in faeces and urine. The plantain lambs excreted a greater proportion of N in faeces than the ryegrass lambs ($621.99 > 52.68\%$), and a smaller percentage of excreted N was excreted in urine ($p<0.01$).

Table 4.6 Least square means of daily urinary nitrogen excretion from sheep by treatment (urine volume (L), urine N concentration (%), N intake (g/d) and N excretion from urine and faeces as proportions of N intake and N excreted). P-values and lsmeans calculated using SAS 9.4-bit version and a repeat measure analysis model using day as the repeated value.

			p-values			
Value	Plantain	Ryegrass	Treatment	ID(Treatment)	Day	Day*Treatment
Urinary N Excretion						
Urine Volume (L)	3.8 ± 0.2	1.8 ± 0.2	<0.0001	0.0036	0.0799	0.0052
Urine N Concentration (%)	0.12 ± 0.1	0.59 ± 0.09	0.0053	<0.0001	<0.0001	<0.0001
N intake (g/d)	21.9 ± 1.4	30.5 ± 1.6	0.0049	0.0369	0.0001	0.0003
N excreted (g/d)	12.9 ± 0.9	19.6 ± 1	0.0015	0.002	0.0593	0.04
% of N intake						
% of N intake Excreted	59.67 ± 2.29	67.37 ± 2.56	0.0602	0.5352	0.011	0.0876
% of N intake excreted in faeces	37.25 ± 2.04	35.78 ± 2.28	0.6447	0.4302	0.0205	0.084
% of N intake excreted in urine	22.42 ± 1.41	31.59 ± 1.57	0.0034	0.6726	0.2794	0.8267
N retained (%)	40.33 ± 2.29	32.63 ± 2.56	0.0602	0.5352	0.011	0.0876
% of Excreted N						
In faeces	61.99 ± 1.7	52.68 ± 1.9	0.0082	0.6425	0.3538	0.3162
In urine	38.01 ± 1.7	47.32 ± 1.9	0.0082	0.6425	0.3538	0.3162

5 Discussion

The purpose of this research was to investigate whether the time of day and feeding either plantain (*Plantago lanceolata* L.) or perennial ryegrass (*Lolium perenne* L.) to lambs would affect creatinine concentration and excretion rate and measure the *in vivo* digestibility of the two forages when fed to lambs.

5.1. Forage effect on urinary excretion

5.1.1. Forage effect on urine volume

The concentrations of creatinine, N, urea, allantoin and GE were all more dilute for the lambs fed plantain. This change in concentration was primarily the result of a diuretic effect of plantain on sheep which drastically increased the mean urine volume in our experiment from 1.8 L for the ryegrass lambs to 3.8 L for the plantain lambs. This represents an increase of approximately 106% in urine volume. This is greater than the 0.5 L/d increase in urine volume reported for six-month-old Romney ewe lambs (O'Connell et al., 2016) and similar to the increase of 1.9 L/d observed in five-month-old Romney-cross lambs when fed plantain compared to ryegrass (McGusty, 2017b). The mechanism of this diuretic effect is not completely understood but a few mechanisms have been proposed. The first is that plantain has a lower DM content than perennial ryegrass, so for a set DM intake, more water is ingested from feed (Minneé et al., 2019). In our experiment, total water intake from forage and via drinking showed a strong positive relationship with urine volume. The plantain fed lambs had the highest water intakes and urine volumes which supports the idea that the lower DM content of plantain can contribute to increased urine volume, though there was still a substantial amount of variation in urine volume unaccounted for. Similarly, in one study where water intake was balanced across treatments, a 0.5 L/d increase in urine volume was found for sheep fed plantain compared to those fed ryegrass (O'Connell et al., 2016). It is likely that a combination of water intake and other factors is responsible for the diuretic effect of plantain which this study cannot identify.

5.1.2. Forage effect on creatinine excretion

One of the main objectives of this experiment was to investigate the creatinine excretion pattern of lambs fed perennial ryegrass or plantain with a focus on the use of creatinine concentration in urine spot-samples to predict the daily volume of urine excreted by sheep. Using creatinine concentration to predict urine volume relies on a couple of assumptions. The first assumption is that the amount of creatinine excreted by an animal daily is the product of liveweight and a fixed coefficient. Our results support this assumption that there is a fixed coefficient as the daily amount of creatinine excreted by the lambs in our experiment in mmol/kg $LW^{0.75}$ was not significantly affected by forage or animal. However, when comparing with the results of other studies there is substantial variation. The mean daily creatinine excretion rate in this experiment for all lambs was 0.324 mmol creatinine per kg $LW^{0.75}$; previous studies with sheep have reported both higher daily creatinine excretion rates of 0.36-0.50 mmol/kg $LW^{0.75}$ (Del Valle et al., 2023; Dos Santos et al., 2018) and lower rates of 0.2-0.23 (David et al., 2015; Del Valle et al., 2023; Yu et al., 2001). The probable reason for this variation is that creatinine is produced in muscle tissue from creatine and that the proportion of muscle in the liveweight of sheep varies by age, sex and breed. An example of this is the differences in creatinine excretion rates from Del Valle et al. (2023) using Santa Inês sheep from multiple trials shown in Table 2.8.

Table 2.8, the lowest creatinine excretion rates of 0.2 and 0.23 mmol/kg $LW^{0.75}$ came from adult sheep, whereas the highest creatinine excretion rate of 0.50 mmol/kg $LW^{0.75}$ came from 3-5 month old lambs of the same breed. This means that while our results support the concept of a fixed rate of creatinine excretion per kg $LW^{0.75}$, this rate varies depending on the sheep used. For this reason, it may be beneficial for research to be undertaken that studies the effect of the age, sex and breed of sheep on daily creatinine excretion rate.

Mean daily creatinine excretion may not have been different between the lambs fed the two forages but the mean creatinine concentration in the urine of the ryegrass sheep (2857 µmol/L) was greater than concentration from the plantain lambs (1324 µmol/L). These concentrations of creatinine measured are within the range found by Jonker et al. (2021) previously. The difference in creatinine concentration can be explained by the

increase in urine volume for the plantain fed lambs whereby the decrease in creatinine concentration is close to being proportional to the increase in urine volume. In this experiment, feeding ryegrass increased the mean creatinine concentration by 116% which is close to the 106% increase in urine volume when feeding plantain. This is promising when it comes to the use of creatinine concentration to predict urine volume because creatinine concentration needs to vary depending on urine volume for the method to work.

5.2. The time of day on urinary excretion

The second assumption used when predicting urine volume from creatinine is that the concentration of creatinine in the spot sample used reflects the ratio of creatinine and urine excreted daily. In our experiment, creatinine concentration varied significantly based on the time of day with higher mean creatinine concentrations observed for both groups in the morning. This variance in the concentration of creatinine of the spot samples could have contributed to the wide range of urine volumes predicted from creatinine concentration by (Maheswaran et al., 2022). The variation in creatinine concentration could be due to variation in the amount of creatinine or volume of urine excreted. For this experiment, it looks likely that a large amount of variation comes from changes in urine output rate, as concentration of creatinine in our experiment was negatively related to the urine output rate in a strong non-linear relationship. The effect of time of day also needs to be investigated further – both in a grazing scenario and with different diets – because a previous study found creatinine concentration to be highest during nocturnal periods when sheep were fed a combination of sorghum silage and concentrate (Dos Santos et al., 2018).

As the concentration of creatinine varies by time of day, it may be beneficial to identify times when creatinine concentration most closely approximates the daily mean concentration. From this experiment, the daily mean concentration of creatinine for both groups (1168 µmol/L for plantain, 2923 µmol/L for ryegrass) was closest to the mean creatinine concentration of the urine from the 12pm-3pm period (1124 µmol/L for plantain, 2828 µmol/L for ryegrass). So, it may be beneficial when estimating urine volume to collect urine spot samples at this time however, the effect of sampling urine at different times of the day in this experiment could be related to the timing of events

such as when lambs were first given newly cut forage each day. The lambs were first given new feed at 9am each morning which is 6 hours prior to 3pm.

5.3. Equations that use creatinine concentration to predict urine volume

Three published equations were applied to the data to compare measured urine volume with urine volume predicted based on creatinine concentrations.

The equation of Jonker et al (2021) tended to overestimate urine volume with most of the predicted urine volumes values being greater than the observed. This trend was observed when all the creatinine concentration samples were used and when only the daily mean creatinine concentrations for lambs from days 2, 3 and 4 were used.

The volumes of urine predicted using the equation from Maheswaran et al (2022) were on average, closest to the urine volumes measured from the lambs in our experiment. This suggests this equation is a more accurate approach when estimating urine volume from creatinine concentration, but it did require additional information in the form of the daily creatinine excretion rate per kg metabolic liveweight. This equation's accuracy requires that the daily creatinine excretion rate used is representative of the lambs used in the experiment. As previously discussed, there have been a broad range of daily creatinine excretion rates reported in sheep, from 0.2 to 0.5 mmol creatinine per kg $LW^{0.75}$. For this equation to be used accurately there is a need for research that looks into the factors of creatinine excretion rate and whether they can be accounted for.

All three equations tended to overestimate the daily volume of urine excreted by the lambs in our experiment; however, this was more pronounced for the Jonker et al (2021) equation. The reason for this equation being inaccurate for our data may be related to the animals used. The Jonker et al. (2021) equation was developed using data from multiple sheep total collection trials that used Romney or composite wethers, fed varying ryegrass, forage brassica and fodder beet diets (Van Lingen et al., 2021). These animals were on average much heavier (41.9 kg > 32 kg), and a couple months older, than the lambs used in our experiment. If these lambs had significantly different body compositions (e.g., more muscle mass) this could explain the overestimated values because the daily creatinine excretion rate could be greater.

The use of the mean daily creatinine concentration from each of the lambs from days 2, 3 and 4 did improve accuracy of the equations. The McGusty (2017b) and Maheswaran et al. (2022) equations were fairly accurate as the trendline equations in Figure 4.6 for predicted urine volume roughly approximate the observed urine volume. This supports both the use of creatinine to predict urine volume in sheep but also the idea that identifying a time of day when creatinine concentration most closely approximates the daily mean concentration could improve accuracy. More research is needed to confirm this as the daily creatinine concentrations of only 11 lambs from 3 days is not a large sample size and vulnerable to sample error.

5.4. Forage digestibility

Digestibility of DM, ash, fat, NDF, ADF and N was not affected by the forage fed to sheep in this experiment. There is very little recent *in vivo* digestibility information for common sheep-grazed forages in New Zealand however, the calculated digestibility values for DM, OM, N, ADF and NDF from the lambs fed perennial ryegrass in this experiment fall within the ranges of previously reported values from a number of European *in vivo* digestibility trials for sheep fed perennial ryegrass (Table 5.1).

Table 5.1. Nutrient digestibility of perennial ryegrass (Lolium perenne L.) fed to sheep from in vivo digestibility trials. (Dry matter, DM; Organic matter, OM; Nitrogen, N; Neutral detergent fibre, NDF; Acid detergent fibre, ADF).

	Digestibility (%)				
	DM	OM	N	NDF	ADF
Spring					
(Niderkorn et al., 2019)	76.7	79.8	68.8	76.6	74.8
(Garry et al., 2020)	80.7-85.8	82.4-87.3	71.3-79	81-85.8	77.8-86.3
(Garry et al., 2021)	75.5-77.9	78.4-81.5	69.2-74	76.3-78.8	
(Hurley et al., 2021)*	81.4	84	78.3	78.7	69.2
(Woodmartin et al., 2024)	79.9	82.1	62.5	64.9	79.1
Summer					
(Beecher et al., 2018)	73.0-76.5	74.8-78.5		73.0-77.2	61.5-63.3
(Garry et al., 2020)	71.5-73.9	73.4-76	66.3-70.7	73.8-75	63.7-73.3
(Garry et al., 2021)	77-78	78.5-80.2	75.3-76.3	70.7-75.4	
(Hurley et al., 2021)*	75.8	78.6	68.5	70.6	64.5
(Yan, 2025)	57	59.1	50.2	51.2	51.3
Autumn					
(Beecher et al., 2018)	71.7-76.4	73.3-78.2		74.8-81.5	61.5-69.1
(Garry et al., 2020)	75.1-78.3	77.1-79.6	78.9-81.3	79.6-83.8	69.5-73.4
(Hurley et al., 2021)*	72.8	75.9	72.3	69.8	65.4
Experiment Ryegrass	77.9	80.2	74	76.7	76.1
Experiment Plantain	81.2	84.5	67.5	80.9	81.1

*Values presented are lsmeans of digestibility of both perennial ryegrass and perennial ryegrass-white clover swards by season.

5.4.1. Dry matter digestibility

Perennial ryegrass in this experiment was calculated to have a mean DM digestibility of 77.9% whilst plantain had a mean DMD digestibility of 81.2%. The mean DM digestibility of perennial ryegrass is similar in value to the 77-78% reported from sheep in summer by Garry et al. (2021) despite the ryegrass in this experiment having a lower CP and higher NDF concentration. Both Garry et al. (2020) and Hurley et al. (2021), reported slightly lower DM digestibility values for summer perennial ryegrass of 71.5-75.8% which could be attributed to lower digestibility values for OM, N and ADF. It is of interest however, that an experiment with some of the same sheep used in this experiment carried out about a month earlier using perennial ryegrass measured much lower digestibility values for DM, OM, N, ADF and NDF; DM digestibility was measured to be

57% (Yan, 2025). The perennial ryegrass in the study by Yan (2025) had a higher DM percentage of approximately 35%, less crude protein (10%) and greater concentrations of NDF, ADF and lignin.

There is little information regarding the *in vivo* digestibility of plantain in sheep, but the DM digestibility calculated for plantain of 81.2% is greater than the 57.3-75.7% estimated for dairy cattle when feeding reproductive and vegetative plantain (Della Rosa et al., 2022). The lack of difference in DM digestibility and lower concentration of DM and NDF in plantain may result in plantain having a faster rate of passage through the rumen, which could allow for greater voluntary feed intakes (Li & Kemp, 2005).

It was hypothesised that plantain would have a lower DM digestibility compared to perennial ryegrass as a previous study estimated both vegetative and reproductive plantain to have a lower DM digestibility when fed to dairy cattle (Della Rosa et al., 2022) and 25% inclusion of plantain in a perennial ryegrass pasture fed to sheep in spring reduced NDF digestibility (Woodmartin et al., 2024). In this experiment, the mean DM digestibility was not different between the forages which suggests that there is no difference in the DM digestibility of perennial ryegrass and plantain fed to sheep in late summer. For the Della Rosa et al. (2022) study, DM digestibility was estimated from the indigestible NDF concentration (determined using *in vitro* methods*)* of the forages used which could explain the difference in plantain DM digestibility. Plantain in both our results and the Della Rosa et al. (2022) study had greater concentrations of lignin, which is typically associated with slower rates of fibre digestion (Chaves et al., 2002). However, DM and NDF digestibility were unaffected, and lignin digestibility was relatively high for both forages.

There is little research on the *in vivo* digestibility of lignin in forages fed to sheep in New Zealand, but it is worth noting that the digestibility of lignin in this experiment is well above the other values that have been reported. Porter and Singleton (1971) reported lignin digestibility from sheep fed hay or oat straw to be between 1.2-10.2% and in another study, acetyl bromide soluble lignin digestibility of perennial ryegrass was measured to be 2.1% (Fahey et al., 1979). More recently, in an experiment carried out about a month earlier than the current experiment and using some of the same lambs, *in vivo* lignin digestibility in summer perennial ryegrass was measured to be 27% (Yan,

2025). These values are much smaller than those measured in this experiment; 46% for ryegrass and 76% for plantain.

The most likely explanation for why the calculated digestibility values for lignin are high is to do with the method of chemical analysis, because lignin is usually considered indigestible (Chaves et al., 2002). The method used to analyse lignin can also influence the measured lignin concentration (Fahey et al., 1979). Common issues with measuring lignin include the partial destruction of lignin by reagents during chemical analysis or by lignin forming soluble complexes with carbohydrates that aren't necessarily found in fibrous residues (Fahey & Jung, 1983). Other issues include non-fibre compounds being included in the fibrous residues. The method used could also explain the difference in lignin digestibility between the forages as lignin composition varies between forages (Raffrenato et al., 2017), and the effect of each method is not consistent between forages (Fahey et al., 1979). Regardless of why the measured lignin digestibility was high, these results suggest that estimations of DM or NDF digestibility using indigestible NDF or lignin may not be accurate for perennial ryegrass and plantain fed to sheep. It may also be of interest for future research to investigate the quality of fibre in these two forages.

5.4.2. Nitrogen digestibility and excretion

Nitrogen utilisation and excretion are of high importance for farming systems. The retention of N is indicative of the efficiency to which N is used, while the N excreted in faeces and urine can represent a loss of N in farming systems and pose a threat to the environment via nitrate leaching or nitrous oxide emissions. One of the main benefits of feeding plantain to ruminants is that it can both dilute and reduce the excretion of N in urine (Nguyen et al., 2022). Digestibility of N was not significantly affected in this experiment by feeding plantain or perennial ryegrass, but the excretion of N was affected.

Firstly, the mean daily excretion of N from the lambs fed plantain was 34.2% lower than the mean daily N excretion of the lambs fed perennial ryegrass. Most of this decrease in daily N excretion could be explained by a 28.2% decrease in N intake for the lambs fed plantain. Plantain typically has lower concentrations of crude protein than perennial ryegrass (Minneé et al., 2019), this was observed in this experiment as well with ryegrass having a greater CP concentration of 12.5% compared to 9.3% for plantain. Mean dry

matter intake and N retention were not different between the sheep fed the two forages so the difference in CP concentration resulted in the plantain sheep having a lower N intake. The decrease in N excretion when feeding plantain is important because reducing the amount of N deposited onto pastures can reduce nitrate leaching and nitrous oxide emissions (Cheng, Judson, et al., 2017). However, farmers are unlikely to use plantain as a monoculture because lambs can have low growth rates (Kemp et al., 2013) and are more likely to use plantain in a mixed sward with other herbs and clovers. Clovers typically have high CP concentrations so herb-clover mixes with plantain will not necessarily reduce N excretion from sheep.

Secondly, the lambs fed plantain partitioned more N to the faeces and less N to urine than the lambs fed ryegrass. On average, 38% of the N excreted by the plantain lambs and 47% of the N excreted by the ryegrass lambs was excreted in urine. When taking the decrease in total N excretion into consideration, urinary N excretion was reduced by 47.2% for the plantain fed lambs compared to those fed perennial ryegrass. Similar reductions in urinary N excretion have been reported when feeding a 100% plantain diet to other ruminants, including 33-37% for dairy cattle (Cheng, Judson, et al., 2017), and 34.9% for red deer (Beck et al., 2020). Smaller reductions in urinary N excretion have also been reported with 30% inclusion of plantain in pasture for dairy cattle (Nguyen et al., 2022).

It has been suggested that plantain has less soluble protein or rumen degradable protein (RDP) than perennial ryegrass (Minneé et al., 2019), which could explain the difference in N partitioning between the faeces and urine. The amount of RDP in the diet of ruminants is positively associated with urinary N excretion (Nennich et al., 2006), so you would expect a decrease in urinary N excretion with less RDP in the diet. Whether plantain had less rumen degradable protein or not cannot be ascertained from the results of this experiment, but it is of note that the experimental mean N digestibility of 67.5% for the plantain, while not significantly different from the mean of 74% for ryegrass, tended to be smaller ($p<0.1$). Importantly, these results demonstrate that less N in the diet of sheep fed plantain is excreted in urine. Urinary N has higher emission factors and is more likely to be leached than faecal N because N in faeces must first be

mineralised before nitrification can occur so reducing the N excreted in urine is one way to approach reducing nitrate leaching and nitrous oxide emissions.

5.5. Experimental design

The experiment was carried out over an 8-day period with a 3-day adaptation period in individual pens and a 5-day collection phase in metabolism crates. This was done to directly measure the individual feed and water input and urine and faecal output of each lamb. The use of metabolism crates and the total collection method is considered accurate (Della Rosa et al., 2021), but limited studies have been carried out with sheep in New Zealand in recent years due to the method being expensive and labour-intensive. Many digestibility studies will use *in vitro* methods which are less dependent on animals but risk being less accurate as digestibility is not directly measured.

Increased concern for animal welfare has also contributed to the shift from metabolism crates. Previously, it has been recommended that total collection digestibility trials with metabolism crates have a collection phase of 4-7 days (Farenzena et al., 2017; Sun et al., 2017), but these recommendations are mostly based on the desired level of precision and don't consider the effect on animal welfare. Isolation stress in metabolism crates can affect an animal's behaviour and feed intake (Bickell et al., 2014), and elevated levels of cortisol have been reported in both wethers (Kapp et al., 1997) and ewes (Abdel-Rahman et al., 2000) when housed in metabolism crates. It is probably inevitable that some stress is induced when using methods that place animals outside of their standard routine, and this could explain why two of the lambs in this experiment had abnormally low feed intakes and subsequently had to be excluded from the digestibility data. Steps were taken to minimise the impact of stress on the animals in the experiment including positioning all the lambs so they could see each other, and providing an empty milk bottle tied to each crate with a piece of string as a form of enrichment so that the lambs had something to interact with. The lambs used in this experiment were also hand reared, so they were used to human contact. Future research using metabolism crates may benefit from measuring stress markers such as faecal cortisol.

6 Conclusions

The pattern of creatinine excretion by lambs was affected by the time of day as there were significant differences in the concentration of creatinine in urine excreted. The strong relationship between creatinine concentration and urine output rate suggests that this may have been influenced by changes in urine output rate, rather than changes in the amount of creatinine excreted. For both forage groups, daily mean creatinine concentration was closest to the mean concentration of creatinine from the 12pm-3pm period. Thus, collecting urine during this time may improve the accuracy of urine volume predictions. For further understanding, research should investigate whether the same pattern in creatinine excretion is observed in grazing sheep or varies depending on when they are eating.

The results of this experiment also show that the digestibility of nutrients in perennial ryegrass and plantain fed to lambs during late summer are similar except for lignin which is more digestible in plantain. The measured lignin digestibility values were very high therefore, when estimating the digestibility of perennial ryegrass or plantain in sheep, lignin may not be a good indicator of DM or NDF digestibility.

7 References

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