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Relationship Between Nitrogen Isotopic Discrimination and the Proportion of Dietary Nitrogen Excreted in Urine by Sheep Offered Different Levels of Dietary Non-Protein Nitrogen

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Urinary nitrogen (N) excretion (UN) as a proportion of N intake (NI; UN/NI) is a major determinant of N excretion from ruminants and could be predicted from the N isotopic discrimination occurring between dietary and animal proteins ($\Delta^{15}N$). This study investigated the usefulness of $\Delta^{15}N$ and other plasma biomarkers to reflect changes in UN/NI from sheep offered different levels of dietary urea. Eighteen Merino rams (age, 1–2 years; live weight, 41 ± 3 kg) were allocated to three dietary N treatments for a N balance study. Treatments were control (C), control + 0.5% urea (C+0.5%), and control + 1.2% urea (C+1.2%) and designed to provide maintenance, maintenance plus an additional 15%, and maintenance plus an additional 33% NI, respectively. The urea effect term was used for one-way ANOVA and regression analysis. As NI increased, the UN and retained N (RN) increased linearly ($p < 0.001$), but UN/NI only increased in treatment C+1.2% compared with C ($p < 0.05$). Plasma $\Delta^{15}N$ was positively and significantly correlated with UN and UN/NI ($r = 0.52$, $p = 0.028$; and $r = 0.68$, $p = 0.002$, respectively) and increased linearly ($p < 0.001$) with the highest values observed in C+1.2%. Urine $\delta^{15}N$ changed linearly between C and C+1.2%, but plasma $\delta^{15}N$ increased quadratically ($p < 0.05$). Plasma urea N increased in a linear way across dietary urea levels ($p < 0.001$). The N isotopic difference between plasma and urine (plasma $\delta^{15}N$–urine $\delta^{15}N$) of C did not vary from either of the other treatments; however, it differed between C+0.5% and C+1.2% ($p < 0.05$). The study confirmed the potential usefulness of plasma $\Delta^{15}N$ to estimate UN/NI from sheep. Moreover, plasma $\delta^{15}N$–urine $\delta^{15}N$ can be proposed as a new biomarker of N excretion from small ruminants. These approaches, however, need to be tested in various study conditions.

Keywords: sheep production, nitrogen utilization, nitrogen isotope, urinary nitrogen, urea supplementation

INTRODUCTION

Ruminants are biologically inefficient utilizers of feed nitrogen (N). In general, dietary N loss from ruminants is 70%–80% (Calsamiglia et al., 2010), which is mainly attributed to urinary N (UN; Dijkstra et al., 2013). While this contributes to inefficient N utilization, it also exacerbates environmental pollution (Galloway and Cowling, 2002; Hristov et al., 2011). Therefore, it is important to reduce UN loss and the proportion of feed N intake (NI), which is partitioned into UN (UN/NI), both of which are used as a proxy to reflect potential N excretion from animal production systems to the environment. Feeding ruminants with high dietary N levels increase UN loss and the ratio of UN/NI (Niu et al., 2016). In contrast, reducing dietary N would reduce UN loss and UN/NI, but this may be economically undesirable for producers because it does not maximize animal performance.

The classical method for evaluating animal N partitioning (i.e., UN loss and UN/NI) is the N balance (NB; MacRae et al., 1993) technique. However, it is difficult for researchers to measure NB precisely, especially for larger numbers of grazing animals (MacRae et al., 1993), as the collection of total urinary and fecal output is required from each animal for several days. Moreover, estimating UN loss and UN/NI in ruminants is difficult due to the complex nature of N metabolism and its interaction with environmental conditions. Earlier studies (Herremans et al., 2019; Mendowski et al., 2020) showed that plasma N isotopic fractionation or discrimination between the animal and its diet (plasma $\Delta^{15}N$; plasma $\delta^{15}N$ – feed $\delta^{15}N$), which occurs during animal digestion and metabolism (Cheng et al., 2011; Cantalapiedra-Hijar et al., 2015), was positively related to N excretion (i.e., UN loss and UN/NI) from large ruminants. Previous studies showed that digestion, rumen function, and metabolism may differ between large and small ruminants (Lapierre and Lobley, 2001; Doreau et al., 2003; Kawashima et al., 2007). Since most cited studies are with large ruminants, more research is still required (Lavery and Ferris, 2021; Khanaki et al., 2021) to explore this relationship, particularly as only three studies can be found (Cheng et al., 2013a; Bernard et al., 2020; Khanaki et al., 2021). The NI measurement is hard to measure in grazing systems, though it is needed to quantify N use efficiency (NUE) and UN/NI. The exploration of using plasma $\Delta^{15}N$ to indicate these parameters needs only the collection of representative feed samples without measuring NI.

This study was conducted to determine if UN and UN/NI can be predicted by plasma $\Delta^{15}N$ in sheep fed pure ryegrass hay with various levels of urea supplementation. We proposed that different urea supplementation could increase NI and microbial crude protein (MCP) synthesis and lead to increased UN and UN/NI, without altering other nutrients involved in sheep N partitioning (**Table 1**).

MATERIAL AND METHODS

Experimental Design

The current study was performed during April and May 2021 at the Dookie campus, the University of Melbourne. All experimental procedures involving sheep were approved by the University of Melbourne Animal Ethics Committee, application number 2015190.1. Eighteen Merino rams (41 ± 3 kg of live weight and age between 1 and 2 years) were used. The live weight and age were equal among treatments. Nine rams were allocated randomly in each period of the 21-day NB study (3 rams/treatment/period). Three dietary treatments were designed to provide increasing NI allowances, as follows: control (C) with maintenance NI allowance; C + 0.5% urea (C +0.5%) with maintenance plus an additional 15% NI allowance; and C + 1.2% urea (C+1.2%) with maintenance plus an additional 33% NI allowance delivered by adding extra N in the feed (**Table 1**).

Urea supplementation was achieved by spraying urea on the ryegrass to increase hay CP level without altering the concentration of other nutrients; one unit of urea was mixed with five units of water before being sprayed on ryegrass hay, and the urea-treated feed was kept and tied in nylon bags, 6 days prior to feeding the sheep. Each treatment was fed for 13 days in individual animal pens followed by 8 days in individual metabolic cages, including 2 days for acclimatization and 6 days as measurement days for an NB study.

Before the study commenced, all animals were grazed on fresh pasture. Pure ryegrass hay was fed to the animal prior to the adaptation days. Ryegrass hay was fed to the animals prior to the adaptation period, and during measurement periods, all offered feed was consumed. Sheep were fed twice per day at 8 a.m. and 4 p.m. (with an identical and restricted dry matter (DM) intake (DMI) of 0.97 kg/day), and drinking water was freely available. Throughout the study, sheep were healthy with no clinical symptoms of any disease.

Animal Measurements

Feed refusals were measured once a day to determine the total DMI for each sheep throughout the study. Feed samples were collected twice per day (at 8 a.m. and 4 p.m.) from each sheep for 6 days at feeding time. Daily urine and feces output were measured at 8.30 a.m. during the measurement days, and subsamples were collected and stored at −20°C. The urine from each sheep was collected into a container that included 225 ml of 10% sulfuric acid (H_2SO_4) prior to collection to keep the urine pH below 3. A bucket with a layer of plastic mesh was placed under the drainage funnel under each metabolism cage (1.2 m × 0.55 m), to allow urine to drain through the mesh, and feces was left on top of the mesh for collection. Blood samples were collected from the jugular vein into 10-ml Li-heparinized evacuated tubes on the last measurement day at 1.30 p.m. Subsequently, plasma was obtained after centrifugation (15 min, 1,200 × *g* at 4°C). Plasma samples were stored at −20°C for further biochemical analysis.

Sample Analytical Methods

Plasma, feed (freeze-dried), and feces (oven-dried) samples were analyzed for $\delta^{15}N$ according to the procedure described by Cheng et al. (2011), using isotope-ratio mass spectrometry (PDZ Europa Ltd, Crewe, UK), that is, the $^{15}N/^{14}N$ ratio in the test sample relative to the $^{15}N/^{14}N$ ratio in the standard (air). The

TABLE 1 | Chemical composition of diets.

Item	Treatment		
	C	C+0.5%	C+1.2%
DM, g/kg	897	883	855
OM, g/kg DM	90	90	90
CP, g/kg DM	120	138	160
NDF, g/kg DM	501	495	480

DM, dry matter; OM, organic matter; CP, crude protein; NDF, neutral detergent fiber.

equation [MJ ME/kg of DM = 0.203 × digestibility of organic DM (DOMD; %) − 3.001] was used to predict the dietary ME content (Ministry of Agriculture, Fisheries and Food, 1990). The average daily live weight (g/day) for each animal was determined as the coefficient of the linear regression of live weight (g) over time (days). Feed subsamples were dried (103°C for 24 h) for determination of DM, while another subsample was oven-dried (at 60°C for 72 h) and ground through a 1-mm sieve for subsequent chemical analyses. Feed samples were analyzed for organic matter (OM; 550°C for 6 h) and neutral detergent fiber (NDF) (Van Soest et al., 1991) using a Fibersac analyzer (Ankom Technology Corporation, Fairport, NY, USA). The Kjeldahl method was used to analyze the N concentration in feed (freeze-dried) and feces (oven-dried), and the N concentration of urine (room temperature thawed urine) was analyzed by the Variomax CN analyzer (Elementar Analyzer Systeme GmbH, Hanau, Germany). The sum of allantoin and uric acid was considered as urinary excretion of purine derivatives (PD). Allantoin and uric acid were measured using colorimetric and uricase (kit No. 685-50; Sigma Chemical Co., St. Louis, MO, USA) methods, respectively, as described by Chen and Gomes (1992). To analyze plasma urea N (PUN) and plasma glucose, enzymatic kinetic methods were used on a Daytona RX Clinical Analyzer (Randox, Nishinomiya, Japan). Two liver enzyme activities, alanine aminotransferase (ALT) and aspartate aminotransferase (AST), were measured using methods according to the International Federation of Clinical Chemistry (IFCC). The retained N (RN) (g/day), NUE (%), and apparent N digestibility (ND; %) were calculated by the following equations, respectively:

$$RN\ \left(\frac{g}{d}\right) = NI\ \left(\frac{g}{d}\right) - UN\ \left(\frac{g}{d}\right) - Facal\ \ N\ \left(\frac{g}{d}\right)$$

$$NUE(\%) = \left[RN\ \left(\frac{g}{d}\right) \div NI\ \left(\frac{g}{d}\right)\right] \times 100$$

$$ND\ (\%) = \left[\left(NI\ \left(\frac{g}{d}\right) - FN\ \left(\frac{g}{d}\right)\right)\ \div NI\ \left(\frac{g}{d}\right)\right] \times 100$$

Statistical Analysis

The statistical package of GenStat (version 16; VSN International Ltd., Hemel Hempstead, UK) was used for linear and quadratic regressions and one-way ANOVAs. As sheep were the replication unit in this study, the average value for each measured parameter per treatment was used for statistical analysis. The significant linear and quadratic effects were performed using one-way ANOVA with urea as a treatment factor and period as a block. The significant thresholds were set at p-value < 0.05; trends were reported at 0.05 < p-value < 0.10.

RESULTS

Nitrogen Partitioning and Isotopic Discrimination

As shown in **Table 2**, there was no significant quadratic effect; all significant responses were linear in nature. The NI for treatments C, C+0.5%, and C+1.2% were 22.1, 23.5, and 27.6 g/day, respectively, increasing with higher urea supplementation ($p < 0.001$). As NI increased, the UN ($p < 0.001$), manure N ($p < 0.001$), and RN ($p < 0.05$) increased linearly. Treatment C+1.2% excreted the most UN (9.6 g/day), whereas treatment C excreted the least (6.7 g/day; $p < 0.001$). The UN/NI and UN/FN only increased between treatment C to treatment C+1.2% (from 0.30 to 0.35 and 0.8 to 1.1 g/g, respectively; $p < 0.05$). The PUN increased linearly ($p < 0.001$), as dietary non-protein N increased, and it was higher in C+1.2% (5.8 mmol/L). The digestibility of N (i.e., ND% and DMD%), urinary PD, plasma glucose, and NUE were not affected by dietary urea supplementation and did not change across treatments (**Tables 2**, **3**).

Urine $\delta^{15}N$ only changed linearly between C and C+1.2%, but plasma $\delta^{15}N$ altered quadratically ($p < 0.05$) and tended to be significant linearly ($p = 0.097$) between C and C+0.5% (**Table 3**). The plasma $\delta^{15}N$ minus urine $\delta^{15}N$ was significantly different between C+0.5% and C+1.2% ($p < 0.05$). The $\Delta^{15}N$ between urine and diet (urine $\Delta^{15}N$) increased linearly and quadratically ($p < 0.01$), across the increase in the urea levels. Plasma $\Delta^{15}N$ increased linearly ($p < 0.001$), and it was the highest in C+1.2% (8.01‰ compared to 6.84‰ and 7.72‰ in C and C +0.05%, respectively).

Nitrogen Isotopic Discrimination and Plasma Urea Nitrogen Relationships With Nitrogen Partitioning

As shown in **Table 4**, plasma $\Delta^{15}N$ was positively and significantly correlated with UN ($r = 0.52$; $p = 0.028$) and manure N ($r = 0.56$; $p = 0.016$) as well as UN/NI ($r = 0.68$; $p = 0.002$). Despite a lack of correlation with RN ($p > 0.05$), plasma $\Delta^{15}N$ was significantly negatively correlated with NUE ($r = -0.65$; $p = 0.003$). Urine $\Delta^{15}N$ was positively correlated with manure N ($r = 0.47$) and UN/NI ($r = 0.53$) at the same significant level ($p < 0.05$). Plasma $\delta^{15}N$–urine $\delta^{15}N$ was positively correlated with UN/NI ($r = 0.50$; $p = 0.036$) and negatively with NUE ($r = -0.59$; $p = 0.010$). The PUN was also positively correlated with NI ($r = 0.78$; $p < 0.001$), UN ($r = 0.72$; $p < 0.001$), manure N ($r = 0.71$; $p < 0.001$), UN/NI ($r = 0.50$; $p = 0.033$), UN/FN ($r = 0.55$; $p = 0.019$), and RN ($r = 0.53$; $p = 0.025$). Among all abovementioned biomarkers, only urine $\Delta^{15}N$ was correlated with ALT ($r = -0.50$; $p = 0.034$) as a reflection of liver function. Moreover, a positive significant relationship between plasma $\Delta^{15}N$ and urine $\Delta^{15}N$ ($r = 0.79$; $p < 0.001$) as well as urine $\Delta^{15}N$ with PUN ($r = 0.48$; $p = 0.044$) was found.

TABLE 2 | Nitrogen (N) partitioning in sheep offered three different levels of non-protein N.

	Treatment				p-Value	
	C	C+0.5%	C+1.2%	SEM	Linear	Quadratic
Animal #	6	6	6	–	–	–
Starting live weight, kg/sheep	40.7	40.8	40.9	0.80	0.980	0.929
Ending live weight, kg/sheep	44.0	44.4	45.1	0.85	0.519	0.972
Average live weight gain, g/sheep	158	172	199	0.02	0.276	0.843
DMI, kg/sheep/day	0.97	0.97	0.97	0.02	0.936	0.917
ME, MJ/sheep/day	9.65	9.70	9.20	0.35	0.211	0.373
NI, g/sheep/day	22.1	23.5	27.6	0.93	<0.001	0.117
NI, %	2.3	2.4	2.9	0.11	<0.001	0.169
Urine output, kg/sheep/day	1.51	1.60	1.41	0.24	0.701	0.509
UN content, %	0.48	0.51	0.69	0.07	0.009	0.217
UN, g/sheep/day	6.7	7.5	9.6	0.54	<0.001	0.199
Faces output [1], kg/sheep/day	0.99	1.02	1.04	0.06	0.048	0.148
Feces DM, %	32.4	31.5	33.3	1.82	0.641	0.418
FN content, %	2.53	2.59	2.64	0.08	0.209	0.907
FN, g/sheep/day	8.1	8.3	9.1	0.52	0.074	0.532
Manure N, g/sheep/day	14.8	15.8	18.7	0.69	<0.001	0.144
RN, g/sheep/day	7.3	7.6	8.9	0.71	0.042	0.497
UN/NI, g/g	0.30	0.32	0.35	0.02	0.013	0.699
FN/NI, g/g	0.37	0.36	0.33	0.02	0.132	0.722
UN/FN, g/g	0.8	0.9	1.1	0.10	0.027	0.829
DMD, %	66.8	66.8	64.2	1.87	0.181	0.446
ND, %	63.2	64.4	66.9	2.30	0.133	0.722
NUE, %	32.9	32.3	32.0	2.21	0.677	0.930
Urinary PD, mmol/sheep/day	14.5	15.0	14.7	0.80	0.748	0.542

C, control; C+0.5%, control + 0.5% urea; C+1.2%, control + 1.2% urea; DM, dry matter; DMI, dry matter intake; ME, metabolizable energy [MJ ME/kg of DM = 0.203 × digestibility of organic DM (%) – 3.001]; NI, nitrogen intake; UN, urinary nitrogen; FN, fecal nitrogen; Manure N, total N output (UN + FN); RN, retained nitrogen; DMD, dry matter digestibility; ND, nitrogen digestibility; NUE, nitrogen use efficiency: 100 × (RN/NI); PD, purine derivatives.

[1] *As wet basis.*

DISCUSSION

Nitrogen Metabolism and Nitrogen Partitioning

When diets were supplemented with urea to increase NI, which ranged from 22.1 and 23.5 to 27.6 g/day, the UN increased (C, C +0.5%, and C+1.2% excreted 6.7, 7.5, and 9.6 g/day, respectively; **Figure 1**). Moreover, when NI increased, manure N increased, which agrees with studies conducted in lactating dairy cows (Colmenero and Broderick, 2006; Kidane et al., 2018) and in mature rams Khanaki et al., 2021). The UN/NI ranged between 0.3 and 0.4 g/g, which was lower than reported by Brand et al. (1992) and within the range observed in other studies (Bernard et al., 2020; Khanaki et al., 2021), and both UN/NI and UN/FN increased significantly from treatment C to treatment C+1.2%. The positive impact of N content on RN was observed, and non-significant sheep average daily live weight gain (158, 172, and 199 g for C, C+0.5%, and C+1.2%, respectively) may explain in part why RN increased with increasing NI ($p < 0.001$; **Figure 2**). In contrast, with increasing animal age, less protein is retained, and metabolizable protein requirements decline (Institut national de la rechere agronomique (INRA), 2018), which may cause increased UN/NI if protein supply is not adjusted. However, in this study, the increased UN/NI is due to increased dietary non-protein N. The amount of FN did not change with

TABLE 3 | Plasma urea nitrogen (PUN), plasma glucose, and natural enrichment of N-15 in feed, urine, and plasma, and nitrogen isotopic discrimination ($\Delta^{15}N$) of sheep offered three different levels of non-protein N.

	Treatment				p-Value	
	C	C+0.5%	C+1.2%	SEM	Linear	Quadratic
PUN, mmol/L	4.3	5.0	5.8	0.37	0.001	0.939
Plasma glucose, mmol/L	3.9	3.9	3.9	0.18	1.000	0.917
ALT, U/L	12	6	8	3.0	0.204	0.131
AST, U/L	88	88	86	11.0	0.894	0.925
Feed $\delta^{15}N$, ‰	1.92	0.45	0.39	–	–	–
Urine $\delta^{15}N$, ‰	0.34	0.00	–0.40	0.31	0.028	0.917
Plasma $\delta^{15}N$, ‰	8.76^{a}	8.17^{b}	8.40^{ab}	0.21	0.097	0.037
Plasma $\delta^{15}N$–urine $\delta^{15}N$, ‰	8.42^{ab}	8.17^{b}	8.80^{a}	0.22	0.108	0.039
Urine $\Delta^{15}N$, ‰	-1.58^{b}	-0.45^{a}	-0.79^{a}	0.26	0.008	0.005
Plasma $\Delta^{15}N$, ‰	6.84	7.72	8.01	0.19	<0.001	0.095

C, control; C+0.5%, control + 0.5% urea; C+1.2%, control + 1.2% urea; PUN, plasma urea nitrogen; ALT, alanine aminotransferase; AST, aspartate aminotransferase.

TABLE 4 | Pearson's correlation coefficients (r) between plasma nitrogen (N) isotopic discrimination (plasma $\Delta^{15}N$), urine $\Delta^{15}N$, and plasma urea N (PUN) with N partitioning variables in sheep.

	Plasma $\Delta^{15}N$, ‰	Urine $\Delta^{15}N$, ‰	Plasma $\delta^{15}N$–urine $\delta^{15}N$, ‰	PUN, mmol/L
DMI, kg/day	0.03	–0.14	0.20	–0.10
NI, g/day	0.24	0.28	0.10	0.78***
UN, g/day	0.52*	0.46	0.33	0.72***
FN, g/day	0.36	0.28	0.26	0.36
UN/NI, g/g	0.68**	0.53*	0.50*	0.50*
FN/NI, g/g	0.10	–0.03	0.17	0.45
UN/FN, g/g	0.33	0.35	0.17	0.55*
Manure N, g/day	0.56*	0.47*	0.37	0.71***
NUE, %	–0.65**	–0.41	–0.59*	0.02
RN, g/day	–0.28	–0.10	–0.35	0.53*
ND, %	–0.10	0.03	–0.17	0.45
DMD, %	–0.20	–0.14	–0.20	–0.20
Urinary PD, mmol/day	0.12	0.04	0.14	0.03
ALT	–0.22	–0.50*	0.17	–0.24
AST	0.01	–0.05	0.17	0.07
Plasma glucose, mmol/L	0.51	0.51	0.37	0.41
PUN, mmol/L	0.36	0.48*	0.05	–
Urine $\Delta^{15}N$, ‰	0.79***	–	0.14	0.48*

PUN, plasma urea nitrogen; DMI, dry matter intake; NI, nitrogen intake; UN, urinary nitrogen; FN, fecal nitrogen; NUE, nitrogen use efficiency = 100 × (RN/NI); RN, retained nitrogen; ND, nitrogen digestibility; DMD, dry matter digestibility; PD, purine derivatives; ALT, alanine aminotransferase; AST, aspartate aminotransferase.
**p-value < 0.05; **p-value < 0.01; ***p-value < 0.001.*

increasing NI. This result is consistent with the other reports (Niu et al., 2016; Kidane et al., 2018), which showed that as animals consume more N, there is less of an increase in FN than UN. The explanation for finding a non-significant dietary impact on NUE might be due to the methodological limitation of NB studies. Spanghero and Kowalski (1997) indicated that as NI increased, the errors (overestimation) of NB increased. Inconsistent with study results by Ferris et al. (1999) in dairy cows, by adding urea supplementation into the diet in the current study, ND% was not significantly different among treatments.

Nitrogen Isotopic Discrimination and Plasma Urea Nitrogen in Relation to Nitrogen Partitioning

Plasma $\Delta^{15}N$ needs a representative feed sample, which can be hard to achieve in grazing systems. Use of plasma $\delta^{15}N$–urine $\delta^{15}N$ requires no feed samples, as long as representative urine samples can be obtained (e.g., multiple urine samples from cows at milking). To the best of our knowledge, this is the first study to introduce plasma $\delta^{15}N$–urine $\delta^{15}N$ as a new biomarker to detect N excretion from ruminants.

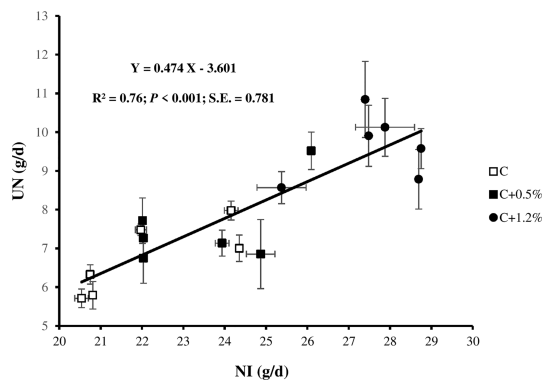

FIGURE 1 | Relationship between urinary nitrogen (UN) and N intake (NI) for individual observations of the sheep offered three different levels of non-protein N: C, control dietary treatment with maintenance NI allowance; C +0.5%, control dietary treatment with maintenance plus an additional 15% NI allowance; C+1.2%, control dietary treatment with maintenance plus an additional 33% NI allowance. The error bars show SE.

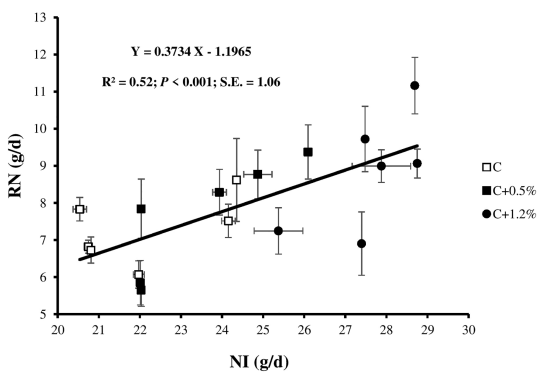

FIGURE 2 | Relationship between retained nitrogen (RN) and N intake (NI) for individual observations of the sheep offered three different levels of non-protein N: C, control dietary treatment with maintenance NI allowance; C +0.5%, control dietary treatment with maintenance plus an additional 15% NI allowance; C+1.2%, control dietary treatment with maintenance plus an additional 33% NI allowance. The error bars show SE.

On average, plasma was enriched in ^{15}N by 7.52‰, while urine was depleted in ^{15}N by 0.94‰ relative to the diet (**Table 3**). Most of the N in plasma is true protein, which was previously shown to be enriched in ^{15}N (Cheng et al., 2010; Cheng et al., 2016). In contrast, the main source of N in the urine is urea, which is reported to be depleted in ^{15}N (Steele and Daniel, 1978; Cheng et al., 2016). The range of plasma $\Delta^{15}N$ values in this study is higher than the range stated in previous studies (Cheng et al., 2013a; Cheng et al., 2016; Bernard et al., 2020; Khanaki et al., 2021). We have no explanation for the differences between these previous studies and our current study, as all sheep were healthy and unstressed throughout the experiment. However, unclear biological or physiological reasons may cause the different plasma $\delta^{15}N$ values (Lee et al., 2012). Nonetheless, the higher plasma $\delta^{15}N$ led to the higher plasma $\Delta^{15}N$. In this study, plasma $\Delta^{15}N$ reflected N partitioning including UN ($r = 0.52$; $p < 0.05$; **Figure 3**), UN/NI ($r = 0.68$; $p < 0.01$; **Figure 4**), and manure N. The results are consistent with the results of other studies (Cheng et al., 2013a; Bernard et al., 2020), as they earlier confirmed the usefulness of using plasma $\Delta^{15}N$ for estimating N excretion (i.e., UN and UN/NI) from small ruminants.

The urine $\Delta^{15}N$ value for treatment C was lower than the range, and for treatments C+0.5% and C+1.2%, urine $\Delta^{15}N$ values were in the range reported by Cheng et al. (2016). Plasma $\delta^{15}N$–urine $\delta^{15}N$ was also positively correlated with UN/NI, but this relationship was not reported in any other NB studies. This result indicated the potential use of this proxy to estimate N excretion, without the need to know the composition of dietary intake. The positive correlations between plasma $\delta^{15}N$ and urine $\delta^{15}N$ (**Figure 5**) are consistent with one of our previous studies (unpublished data). Kohn et al. (2005) showed the potential for using PUN as an appropriate biomarker to evaluate N partitioning. In the present study, PUN concentrations were also related to the difference in N partitioning (i.e., NI, UN, UN/NI, UN/FN, manure N, and RN) in C+1.2% compared to the other two treatments and were within the range described by Kohn et al. (2005).

Previous studies (Sick et al., 1997; Cheng et al., 2011; Cantalapiedra-Hijar et al., 2015) have shown that plasma $\Delta^{15}N$ originates from animal digestion (i.e., rumen site) and metabolism (i.e., liver site). Moreover, Zuntz (1981) suggested that rumen microflora have the capabilities to break down cellulose (as an energy source) and convert non-protein N (i.e., urea) to microbial protein. However, in the current study, offering different NI to the animals and similar urinary PD results across treatments could be interpreted as a lower efficiency of MCP synthesis. The explanations for this result are as follows: 1) the sheep were fed marginally above their requirement, possibly explaining the limited change in MCP and 2) the fact that productivity and efficiency do not always go together. For instance, a greater supply of MCP leads to greater milk protein yield or live weight gain but lower MCP efficiency use (National Research Council (NRC), 2001; Institut national de la recherche agronomique (INRA), 2018). In general, and when talking about N whether in digestion or metabolism, increased supply translates to higher productivity but lower efficiency.

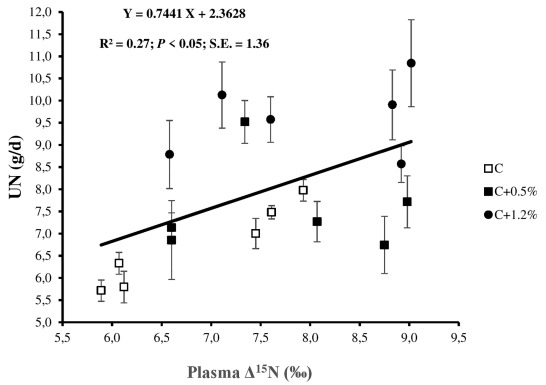

FIGURE 3 | Relationship between urinary nitrogen (N; UN) and plasma N isotopic discrimination (plasma $\Delta^{15}N$) for individual observations of the sheep offered three different levels of non-protein N: C, control dietary treatment with maintenance N intake (NI) allowance; C+0.5%, control dietary treatment with maintenance plus an additional 15% NI allowance; C+1.2%, control dietary treatment with maintenance plus an additional 33% NI allowance. The error bars show SE.

Nitrogen Utilization in Relation to Nitrogen Isotopic Discrimination

The less efficiently the animals use dietary N, the higher the plasma $\Delta^{15}N$ (Sick et al., 1997; Cheng et al., 2013a and Cheng et al., 2013b; Cantalapiedra-Hijar et al., 2018). Some other studies (Cheng et al., 2011; Bernard et al., 2020; Khanaki et al., 2021) showed that the NUE decreased when NI increased. However, in the present study, NUE did not change when NI increased from treatment C to treatment C+1.2%. There are three possible reasons for this: 1) the variation in NI among treatments was insufficient to differentiate NUE; 2) the sheep were fed marginally above their requirement,

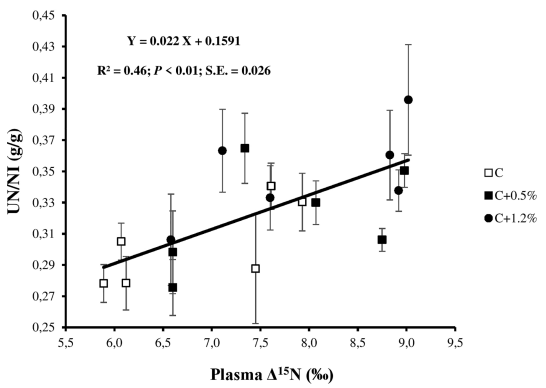

FIGURE 4 | Relationship between urinary nitrogen (N; UN) to N intake (NI) ratio (UN/NI) and plasma N isotopic discrimination (plasma $\Delta^{15}N$) for individual observations of the sheep offered three different levels of non-protein N: C, control dietary treatment with maintenance N intake (NI) allowance; C+0.5%, control dietary treatment with maintenance plus an additional 15% NI allowance; C+1.2%, control dietary treatment with maintenance plus an additional 33% NI allowance. The error bars show SE.

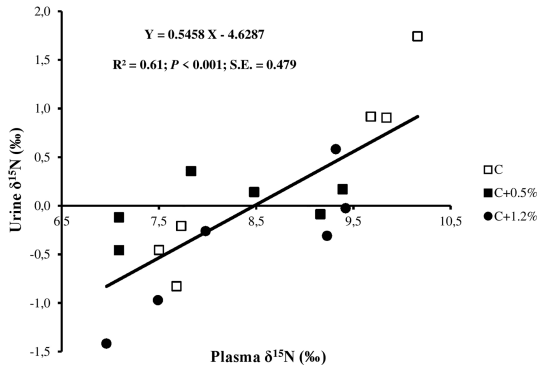

FIGURE 5 | Relationship between urine $\delta^{15}N$ and plasma $\delta^{15}N$ for individual observations of the sheep offered three different levels of non-protein N: C, control dietary treatment with maintenance N intake (NI) allowance; C+0.5%, control dietary treatment with maintenance plus an additional 15% NI allowance; C+1.2%, control dietary treatment with maintenance plus an additional 33% NI allowance. The error bars show SE.

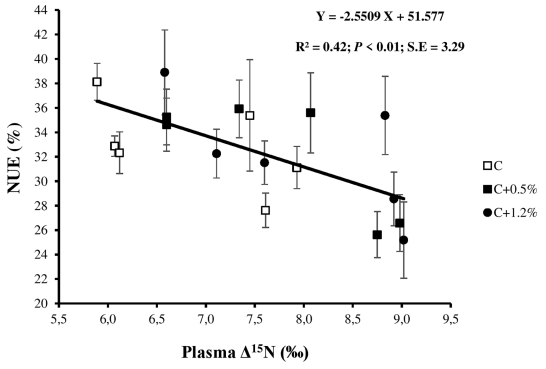

FIGURE 6 | Relationship between nitrogen (N) use efficiency (NUE) and plasma N isotopic discrimination (plasma $\Delta^{15}N$) for individual observations of the sheep offered three different levels of non-protein N: C, control dietary treatment with maintenance N intake (NI) allowance; C+0.5%, control dietary treatment with maintenance plus an additional 15% NI allowance; C+1.2%, control dietary treatment with maintenance plus an additional 33% NI allowance. The error bars show SE.

possibly explaining the inability to differentiate NUE; and 3) the errors of NB increased when NI increased. Spanghero and Kowalski (1997) illustrated the difficulties of the NB technique and its tendency to overestimate RN, even when well conducted. A negative significant relationship between NUE and plasma $\Delta^{15}N$ ($p < 0.01$; **Figure 6**) is similar to the other studies' reports (Cheng et al., 2013a; Bernard et al., 2020; Khanaki et al., 2021) and the result of a meta-analysis (Cantalapiedra-Hijar et al., 2018).

Nitrogen Excretion in Relation to Nitrogen Isotopic Discrimination

Plasma $\Delta^{15}N$ correlation with NI was low, which suggested that plasma $\Delta^{15}N$ was not highly impacted by NI in the current study. There was a significant relationship between UN and plasma $\Delta^{15}N$ ($r = 0.52$), as the animals ate the same diet and differentiated only in NI through urea supplementation. The result is consistent with previous studies by Bernard et al. (2020) and Khanaki et al. (2021), suggesting that plasma $\Delta^{15}N$ is more related to UN rather than NUE, likely because some of the mechanisms underlying the latter (i.e., N mobilization) do not fractionate N isotopes. A positive significant relationship between UN/NI and plasma $\Delta^{15}N$ (**Figure 4**) is consistent with the results of other studies (Cheng et al., 2011; Cheng et al., 2013a; Bernard et al., 2020; Khanaki et al., 2021). This emphasized the potential to use plasma $\Delta^{15}N$ to estimate UN/NI, which is hard to measure in in-field conditions (Spanghero and Kowalski, 1997), especially under production systems of grazing ruminants (Cheng et al., 2018). Moreover, the ND and DMD relationships with plasma $\Delta^{15}N$ ($r = -0.10$ and $r = -0.20$, respectively) were low, suggesting that the effect of the overall digestion process on the relationship between UN/NI and $\Delta^{15}N$ is limited.

CONCLUSIONS

As NI increased by adding more non-protein N to the diet, the UN and UN/NI increased, but the NUE had a limited response to NI in this study. The change in UN and UN/NI was reflected by plasma $\Delta^{15}N$ changes. The results support the view that the efficiency of N use in the rumen was highly contrasted across treatments and may have a role in the observed plasma $\Delta^{15}N$. Positive and negative linear relationships between UN/NI and NUE with plasma $\Delta^{15}N$, respectively, were observed. The study showed the potential use of plasma $\delta^{15}N$–urine $\delta^{15}N$ to quantify N excretion and NUE from sheep. Moreover, the results demonstrated that plasma $\Delta^{15}N$ works better than the other available biomarkers for reflecting changes in N partitioning, including both N excretion and the efficiency of N use from the ruminants. Further research is required to explore these relationships in sheep of different physiological statuses and offered diets differing widely in N.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

ETHICS STATEMENT

The animal study was reviewed and approved by The University of Melbourne Animal Ethics Committee.

AUTHOR CONTRIBUTIONS

HK: conceptualization, methodology, sampling, data analysis, writing—original draft, and revising. RD: supervision, methodology (supporting), data analysis (supporting), editing, and finalizing the paper for submission. BL: supervision, editing,

and finalizing the paper for submission. GC-H: editing and finalizing the paper for submission. LC: supervision, conceptualization (lead), methodology (supporting), data analysis (supporting), writing—review and editing, and finalizing the manuscript for submission. All authors contributed to the article and approved the submitted version.

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