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The effects of dietary nitrogen to water-soluble carbohydrate ratio on isotopic fractionation and partitioning of nitrogen in non-lactating sheep

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The main objective of this study was to investigate the relationship between partitioning and isotopic fractionation of nitrogen (N) in sheep consuming diets with varying ratios of N to water-soluble carbohydrate (WSC). Six non-lactating sheep were offered a constant dry matter (DM) allowance with one of three ratios of dietary N/WSC, achieved by adding sucrose and urea to lucerne pellets. A replicated 3 dietary treatments (Low, Medium and High N/WSC) × 3 (collection periods) and a Latin square design was used, with two sheep assigned to each treatment in each period. Feed, faeces, urine, plasma, wool, muscle and liver samples were collected and analysed for ^{15}N concentration. Nitrogen intake and outputs in faeces and urine were measured for each sheep using 6-day total collections. Blood urea N (BUN) and urinary excretion of purine derivative were also measured. Treatment effects were tested using general ANOVA; the relationships between measured variables were analysed by linear regression. BUN and N intake increased by 46% and 35%, respectively, when N/WSC increased 2.5-fold. However, no indication of change in microbial protein synthesis was detected. Results indicated effects of dietary treatments on urinary N/faecal N, faecal N/N intake and retained N/N intake. In addition, the linear relationships between plasma $\delta^{15}N$ and urinary N/N intake and muscle $\delta^{15}N$ and retained N/N intake based on individual measurements showed the potential of using N isotopic fractionation as an easy-to-use indicator of N partitioning when N supply exceeds that required to match energy supply in the diet.

Keywords: stable isotope, water-soluble carbohydrates, urinary nitrogen, purine derivatives

Implications

This study showed significant relationships between N isotopic fractionation and N partitioning when N supply exceeds that required to match energy supply in the diet. The difficulties in measuring N partitioning using the N balance technique may be overcome by using N isotopic fractionation, as an easy-to-use indicator of N partitioning. The ease of taking the N isotopic fractionation measurements mean that this approach could be developed for on-farm monitoring of N utilisation, or in breeding programmes for N utilisation.

Introduction

The most widespread method used to measure N partitioning is the N balance (NB) technique. The utilisation of N can be indicated by urea N concentration in blood or milk. However, NB is difficult to conduct, particularly with large numbers of animals (MacRae *et al.*, 1993) and urea N is subject to substantial diurnal variation (Broderick and Clayton, 1997). Earlier studies identified that fractionation of stable N isotopes occurs during digestion and metabolism, notably during utilisation of N by ruminal bacteria fermenting carbohydrates (Wattiaux and Reed, 1995) and metabolism of amino acids in the liver (Sick *et al.*, 1997). Therefore, N isotopic fractionation may potentially be used to quantify N partitioning of ruminants.

Well-managed temperate pasture often provides excess N relative to dietary energy supply (Litherland and Lambert, 2007). This leads to a low efficiency of incorporating feed N into product N (e.g. milk or meat N), and large outputs of surplus N (mainly urine) to the environment. It has been proposed that production and N partitioning (i.e. the distribution of N among feed, urine, faeces, milk and retention in the body) can be manipulated through changing the key dietary compositions, namely the N to water-soluble

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carbohydrate (WSC) ratio in the diet (Miller *et al*., 2001). More feed N is expected to be partitioned to milk rather than urine with a decrease of N/WSC. However, a meta-data analysis and an *in vitro* study demonstrated that there may be an optimum concentration of N and WSC, which optimise rumen fermentation and animal production regardless of changes in N/WSC (Pacheco *et al*., 2007; Burke *et al*., 2011). Hence, the two objectives of this study were to evaluate the use of N isotopic fractionation as an indicator of N partitioning for non-lactating sheep offered three levels of N/WSC in the diet, and to examine the effect of dietary N/WSC on N partitioning.

Material and methods

Six non-lactating sheep aged between 1.5 and 2 years old and weighing 65.0 (s.d. = 3.9), 61.8 (s.d. = 4.4) and 62.5 (s.d. = 3.5) kg at the start of the three collection periods were used. Three dietary treatments (N/WSC) were evaluated in a 3 × 3 Latin square design with three periods of 3 weeks (2 weeks dietary adaptation and 1 week animal measurement in each period). Two sheep were allocated to each treatment in each period.

Diets

All sheep grazed on ryegrass/white clover-based pasture before the study. Lucerne pellets and straw were gradually introduced to the sheep, 10 days before the study commenced. Four days before period 1, sheep were consuming a 100% lucerne pellet diet and were individually housed in metabolism crates. Throughout the study, fresh water was available *ad libitum*.

The diets were designed to provide slightly above maintenance metabolisable energy requirement (11.5 MJ ME/sheep per day; Nicol and Brookes, 2007) with three levels of N/WSC (treatment Low, Medium and High), and expected to generate variations in both N isotopic fractionation and partitioning (Table 1). Additional WSC and N were provided by sprinkling white cane sugar (Chelsea, Auckland, New Zealand; 1700 kJ/100 g) or spraying liquid urea (Biolab, Australia; 46.7% N) onto the lucerne pellets (Winslow Ltd, Ashburton, New Zealand). The diets were formulated by adding 20 to 100 g sucrose/kg DM and 10 to 30 g urea/kg DM to the purchased lucerne pellet, depending on the pre-determined WSC and N concentrations from each batch of pellets.

Animal measurements

This study was undertaken at the Johnstone Memorial Laboratory, Lincoln University, New Zealand, under the authority of Lincoln University Animal Ethics Committee (approval no. 339). Sheep were housed separately in metabolism crates with feed offered once a day at 1000 h. All sheep were weighed on the day before and after each NB period.

Each sheep was offered 1.5 kg (fresh weight) of pellets daily and feed refusals were recorded to calculate dry matter intake (DMI). Feed samples from each treatment were collected for 7 days. Faeces and urine output were measured and sampled during days 2 to 7 of each NB period. A plastic bucket with a layer of metal mesh was placed under the drainage channel of each metabolism crate to allow urine to drain through the mesh and faeces stay on top of the mesh. To keep urine pH between 2 and 3, 250 ml of sulphuric acid (10% vol/vol) was added into each bucket daily before collection.

Blood samples were collected from the jugular vein into 10 ml Li-heparinised evacuated tubes at 1130 h on days 3 and 6 of each NB period. Plasma was immediately harvested by centrifugation at 4°C at 1200 × **g** for 15 min. Gluteal muscle and liver biopsy samples (0.5 g each) were taken from each animal at 0900 h on the last day of each period (Miller-Graber *et al*., 1991). A 30 cm × 30 cm patch of wool from each sheep was shaved off at the beginning of period 1. The regrowth of wool from this patch was harvested 14 days after each NB period (Rogers and Schlink, 2010). All samples were stored at −20°C and pooled per sheep per period before chemical analysis. Retained N (RN) was calculated using the equation: [RN (g/day) = N intake (NI; g/day) − faecal N (FN; g/day) = urinary N (UN; g/day)].

Table 1 *Feed intake and diet composition for the three diets varying in formulated N/WSC*[1]

	Low	Medium	High	*n*	s.e.d.	L[2]	Q[3]
ME (MJ/kg DM)	11.5	10.6	10.4	15	0.07	–	–
WSC concentration (% DM)	22.4	13.0	12.0	15	1.14	–	–
N concentration (% DM)	2.44	2.76	3.25	15	0.017	–	–
N/ME (g/MJ)	2.13	2.60	3.14	15	0.033	–	–
N/WSC (g/g)	0.11	0.22	0.28	15	0.018	–	–
DMI (kg/day)	1.33	1.35	1.35	15	0.019	0.28	0.76
ME intake (MJ/day)	15.3	14.3	14.0	15	0.25	0.003	0.17
DM digestibility (% of DM)	60.9	61.5	57.5	15	0.88	0.01	0.03
N digestibility (% of N)	54.1	64.3	63.8	15	1.34	<0.001	0.01

N = nitrogen; WSC = water-soluble carbohydrate; ME = metabolisable energy; DM = dry matter.
[1]Diets based on: Low (0.11 g N/g WSC); Medium (0.22 g N/g WSC); High (0.27 g N/g WSC).
[2]Linear effects.
[3]Quadratic effects.

Analytical methods

All samples were freeze-dried and analysed for δ^{15}N following the procedure described by Cheng *et al.* (2011) using isotope ratio mass spectrometry (PDZ Europa Ltd, Crewe, UK). *In vitro* digestibility of organic matter on DM basis (DOMD) was measured following the procedure described by Clarke *et al.* (1982). The ME content of feed was calculated using the equation: [MJ ME/kg DM = DOMD (g/kg DM) × 0.016]. Dry matter digestibility (DMD) was calculated using the equation: [DMD (%) = 1 − faecal output (FO; g/day)/DMI (g/day)]. Nitrogen digestibility (ND) was calculated using the equation: [ND (%) = 1 − FN (g/day)/NI (g/day)]. The concentration of WSC in feed samples was analysed following the method described by Rasmussen *et al.* (2007). Feed, urine and faeces were analysed for N concentration using a Variomax CN Analyser (Elementar Analysensysteme GmbH, Hanau, Germany). Blood urea N (BUN) was analysed using an enzymatic kinetic method on a COBAS MIRA clinical analyzer (Roche Diagnostics, Rotkreuz, Switzerland). Urinary excretion of purine derivatives (PD) was analysed following the method described by George *et al.* (2006) using HPLC (Agilent 1100 series, Waldbronn, Germany).

Statistical analysis

The Genstat statistical package (version 12.2; VSN International Ltd) was used for general ANOVA and linear regression analysis. All measured data were combined into single mean values for each sheep per period. The significance of linear and quadratic effects was determined using general ANOVA with N/WSC as treatment factor, and period + sheep as block. *P*-values >0.05 were declared as statistically non-significant.

Results

Three sheep refused feed from the start of the collections in periods 2 (two sheep) and period 3 (one sheep); and one liver sample from a sheep in period 1 was not obtained. Therefore, corresponding measurements were treated as missing values in subsequent statistical analysis.

Nitrogen isotopic fractionation in relation to nitrogen partitioning

Wool, muscle, liver, plasma and faeces were enriched in ^{15}N compared with feed; and urine was depleted in ^{15}N relative to feed (Table 2). Dietary treatments had no significant effects on N isotopic fractionation apart from faecal δ^{15}N − feed δ^{15}N ($P < 0.05$; Table 3). Linear relationships using individual animal data per period were found between plasma δ^{15}N and UN/NI (equation (1) and muscle δ^{15}N and RN/NI (equation (2)).

$$\text{Plasma } \delta^{15}\text{N (‰)} = 3.73 \times \text{UN/NI (g/g)} + 3.53 \quad (n = 15; R^2 = 0.62; P < 0.001; \text{s.e.} = 0.24) \quad (1)$$

$$\text{Muscle } \delta^{15}\text{N (‰)} = -5.26 \times \text{RN/NI (g/g)} + 5.89 \quad (n = 15; R^2 = 0.60; P < 0.001; \text{s.e.} = 0.46) \quad (2)$$

Table 2 *Nitrogen-15 concentrations (delta units expressed relative to standard air; ‰) in the diet, excreta and tissue of sheep offered one of three diets varying in N/WSC*[1]

	Low	Medium	High	*n*	s.e.d.	L[2]	Q[3]
Feed δ^{15}N	−0.03	0.11	−0.01	15	0.087	0.81	0.13
Urine δ^{15}N	−1.72	−1.50	−1.60	15	0.256	0.65	0.52
Faecal δ^{15}N	3.74	2.91	3.13	15	0.180	0.03	0.02
Wool δ^{15}N	4.01	3.94	4.15	15	0.399	0.74	0.71
Plasma δ^{15}N	5.59	5.74	5.65	15	0.071	0.40	0.12
Liver δ^{15}N	5.75	5.87	6.01	14	0.123	0.10	0.98
Muscle δ^{15}N	5.74	5.52	5.71	15	0.194	0.88	0.28

N = nitrogen; WSC = water-soluble carbohydrate.
[1]Diets based on: Low (0.11 g N/g WSC); Medium (0.22 g N/g WSC); High (0.27 g N/g WSC).
[2]Linear effects.
[3]Quadratic effects.

Feed intake and composition

Table 1 shows that DMI was not affected by the dietary treatments. As N/WSC increased from low to high treatment, the ME and ME intake decreased. Treatment effects were observed for ND (% of N) and DMD (% of DM).

Nitrogen partitioning, BUN and PD

Nitrogen intake increased from 32.5 to 44.0 g/day as N/WSC increased (Table 4). Dietary treatment had a linear effect on NI (g/day), urine output (UO, kg/day), UN (g/day), FO (g/day), UN/FN (g/g), FN/NI (g/g) and BUN (mM/l; Table 4). On average, RN was 0.57 g/day and ranged between −7.02 and 6.65 g/day.

Discussion

Nitrogen isotopic fractionation

The average feed δ^{15}N in the current study was 0.05‰ (Table 2), which is similar to the value reported for lucerne (0.00‰) by Steele *et al.* (1983). Faeces, wool, plasma, muscle and liver were on average enriched in δ^{15}N by 3.13, 4.14, 5.70, 5.78 and 7.89‰, respectively, compared with feed, and urine was depleted in δ^{15}N by 1.54‰ relative to feed (Table 3). These findings agree with the results of others (Koyama, 1985; Sponheimer *et al.*, 2003). In addition, the enrichment of wool in the current study was comparable to that previously reported by Männel *et al.* (2007) with sheep grazing pure C_3 sward. Some ecology studies (Hobson *et al.*, 1993; Sponheimer *et al.*, 2003), suggested that body tissues with slower N turnover rates (e.g. muscle and wool) are less likely to reflect N metabolic changes compared with active tissues (e.g. liver and plasma) when feeding studies have short periods. This finding is supported by the low correlation between δ^{15}N of wool and plasma ($R^2 = 0.20$; $P = 0.053$); but was not the case for muscle δ^{15}N, which was well correlated with plasma δ^{15}N ($R^2 = 0.78$; $P < 0.001$). These differences in correlations may be due to differences in the enzymatic discrimination reactions or/and the turnover rates involved in wool and muscle synthesis under current feeding system.

Table 3 *Nitrogen isotopic fractionation of different body N sinks for sheep offered one of three diets varying in N/WSC*[1]

	Low	Medium	High	*n*	s.e.d.	L[2]	Q[3]
Urine δ^{15}N − feed δ^{15}N	−1.69	−1.62	−1.58	15	0.266	0.72	0.94
Faecal δ^{15}N − feed δ^{15}N	3.77	2.80	3.14	15	0.193	0.02	0.01
Plasma δ^{15}N − feed δ^{15}N	5.62	5.63	5.67	15	0.110	0.71	0.87
Liver δ^{15}N − feed δ^{15}N	5.77	5.76	6.07	14	0.162	0.14	0.30
Wool δ^{15}N − feed δ^{15}N	4.04	3.83	4.16	15	0.451	0.81	0.52
Muscle δ^{15}N − feed δ^{15}N	5.77	5.41	5.72	15	0.206	0.81	0.12
Plasma δ^{15}N − urine δ^{15}N	7.31	7.24	7.25	15	0.248	0.82	0.87
Liver δ^{15}N − urine δ^{15}N	7.45	7.36	7.68	14	0.158	0.22	0.21

N = nitrogen; WSC = water soluble carbohydrate.
[1]Diets based on: Low (0.11 g N/g WSC); Medium (0.22 g N/g WSC); High (0.27 g N/g WSC).
[2]Linear effects.
[3]Quadratic effects.

Table 4 *Nitrogen partitioning, blood urea N and urinary excretion of purine derivatives for sheep offered one of three diets varying in N/WSC*[1]

	Low	Medium	High	*n*	s.e.d.	L[2]	Q[3]
N intake (g/day)	32.5	37.2	44.0	15	0.58	<0.001	0.09
Urinary N (g/day)	17.7	21.5	26.8	15	0.64	<0.001	0.22
Urine output (kg/day)	1.43	1.74	2.04	15	0.074	<0.001	0.93
Urinary N concentration (%)	1.26	1.25	1.34	15	0.066	0.28	0.45
Faecal N (g/day)	14.9	13.2	15.8	15	0.69	0.23	0.014
Faecal output (g/day)	521	519	575	15	18.3	0.03	0.13
Faecal N concentration (% of DM)	2.86	2.54	2.76	15	0.075	0.21	0.01
Retained N (g/day)	−0.13	2.53	1.37	15	0.825	0.13	0.04
Urinary N/faecal N (g/g)	1.19	1.63	1.70	15	0.084	0.002	0.054
Urinary N/N intake (g/g)	0.55	0.58	0.61	15	0.022	0.052	0.92
Faecal N/N intake (g/g)	0.46	0.36	0.36	15	0.013	<0.001	0.01
Retained N/N intake (g/g)	−0.01	0.07	0.03	15	0.019	0.09	0.02
BUN (mM/l)	6.36	7.56	9.30	15	0.400	<0.001	0.47
Urinary purine derivatives (mM/day)	33.1	30.9	32.1	15	2.92	0.75	0.53

N = nitrogen; WSC = water-soluble carbohydrate; DM = dry matter; BUN = blood urea N.
[1]Diets based on: Low (0.11 g N/g WSC); Medium (0.22 g N/g WSC); High (0.27 g N/g WSC).
[2]Linear effects.
[3]Quadratic effects.

Nitrogen isotopic fractionation in relation to nitrogen partitioning

Sick *et al*. (1997) provided preliminary evidence in their study with rats that urea and protein from plasma were, respectively, less depleted and more enriched in δ^{15}N compared with feed when NI increased. In general, UN/NI is positively related to NI of the animal (Castillo *et al*., 2001; Cheng *et al*., 2011). Furthermore, the body protein pool and UN are mainly derived from the plasma amino acids and plasma urea pools, respectively (Sick *et al*., 1997). Consequently, we anticipated a negative relationship between muscle δ^{15}N and RN/NI. Similarly, we predicted a positive relationship between plasma δ^{15}N and UN/NI. Our data provided support for these relationships (equations (1) and (2)), with both relationships being statistically significant. It is notable that these relationships occurred even when animals were losing weight (equation (2)).

Wattiaux and Reed (1995) reported that incorporation of ammonia into bacterial protein resulted in depletion and enrichment of ^{15}N in microbial protein and ruminal ammonia, respectively, which would lead to the opposite relationships to those described above (Cheng *et al*., 2011). It seems likely that there was little difference in N isotopic fractionation in the rumen as dietary treatments had no effect on PD excretion in the current study (Table 4), which may indicate that there was no difference in rumen microbial protein synthesis (Cheng *et al*., 2011). The possibility of N turnover leading to ruminal N isotopic fractionation without altered microbial protein synthesis cannot be excluded. as RN/NI and UN/NI increased and decreased, respectively, over the course of the study, it is not possible to preclude the possibility that the relationships described in equations (1) and (2) were due to a residual effect of the diet offered before the study, which may have influenced the ^{15}N content of body protein reserves. However, this seems unlikely as the use of plasma rather than whole blood in the current study would minimise the residual effect. Hilderbrand *et al*. (1996) showed it took less time for plasma (~10 days) from bears

to reach plateau ^{15}N levels compared with red blood cells (~30 days). In addition, Steele and Daniel (1978) showed expected levels of enrichment of plasma and depletion of urine after a 21-day dietary adaptation in cows. Therefore, the previous diet before starting the next collection period would most likely being cleared from body protein and plasma protein in the current study.

Although N isotopic discrimination may occur at various sites in the body, the liver is considered as one of the key sites where the major discrimination occurs in animals (Sick *et al*., 1997). It is possible that more than one N metabolic pathway is involved in the discrimination of N and resulted in the correlations described in equations (1) and (2). However, as an alternative pathway, the level of urea recycling from current diet is expected to be low when NI is greater than requirement (Reynolds and Kristensen, 2008). Therefore, under the condition of this study, the relationship between N isotopic fractionation and N partitioning were mainly driven by deamination or transamination in the liver, as suggested by Macko *et al*. (1986), similar to the fractionation reported by Sick *et al*. (1997) for rats offered a range of protein sources.

Nitrogen metabolism

A similar amount of DMI was consumed by the sheep at all three levels of N/WSC, which eliminated variation in DMI as a factor influencing the dietary effect on N partitioning. The changes in N partitioning are thus expected to be solely a result of the different diet composition. The proportion of NI excreted in urine (UN/NI) in the current study (0.55 to 0.61 g/g) was slightly higher than that reported (0.55 g/g) by Brand *et al*. (1992). The results in Table 4 show a trend ($P < 0.1$) for decreasing UN/NI as N/WSC decreased, similar to Edwards *et al*. (2007) who concluded that a reduction in UN/NI of dairy cows should be expected when N/WSC (g/g) decreased from 0.27 to 0.12. We reasoned that the excessive N supply (the average NI of our sheep was at least 1.8 times the maintenance N requirement according to Brookes and Nicol, 2007) and the relative low fermentable ME limited the microbial protein synthesis resulting in limited treatment effects on UN/NI (van Vuuren *et al*., 1993). This argument is supported by urinary PD excretion (Table 4), suggesting no change in microbial protein synthesis across treatments.

The partitioning of surplus N away from urine to faeces may contribute to reduce nitrate leaching to the ground water and ammonia volatilisation to atmosphere (Varel *et al*., 1999), as well as a reduction in nitrous oxide, which is a potent greenhouse gas. Similar to results reported from a study with dairy cows (Moorby *et al*., 2006), our study showed that UN/FN decreased by around 43% when WSC was added to the diet (Table 4). This change in N excretion pattern was achieved through decreasing NI, UN and UO rather than increasing rumen microbial protein synthesis, which was showed by Miller *et al*. (2001).

BUN is used as an indicator of N utilisation in ruminants (Kohn *et al*., 2005). In this study, BUN results were within the range previously reported (Kohn *et al*., 2005). Using regression analysis, only 13% ($P = 0.1$) of the dietary treatment (N/WSC) variations were explained by changes in individual BUN. The linear relationship between BUN and UN/NI ($R^2 = 0.55$; $P < 0.001$), and BUN and RN/NI ($R^2 = 0.24$; $P = 0.04$) were significant. These low to moderately strong correlations may be due to the changes in urea metabolism and BUN concentrations that were not captured in single spot blood sample (Broderick and Clayton, 1997).

Conclusion

Decreasing N/WSC ratio in the diets led to a reduction in UN/FN. BUN was moderately correlated with UN/NI. Moderate to strong relationships were found between N partitioning and isotopic fractionation when using individual animal data per period, which confirmed the potential to use N isotopic fractionation as an indicator of N partitioning in non-lactating sheep when N supply exceeds that required to match energy supply in the diet. Further studies are required to preclude possible residual effects of the previous diet on the contribution of ^{15}N from body reserves. The rate of N and ^{15}N turnover of different body N sink needs to be determined in order to confirm the use of most appropriate tissue.

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