Ann. Zootech. 48 (1999) 35–46

Original article

In situ degradation of perennial rye grass from grazed pastures during the season at two levels of nitrogen fertilization

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(Received 25 November 1997; accepted 10 June 1998)

Abstract — In situ incbations were carried out to investigate the effect of rate of nitrogen fertilization and season on rumen degradation of ryegrass. Samples of *Lolium perenne* of grazed herbage, fertilized at two levels of nitrogen (250 and 550 kg N/ha/year), were collected in May, July-August, September and October, and incubated in nylon bags in the rumen of three grazing dairy cows with a rumen cannula. Theoretical degradability in the rumen varied between 56 and 64% for organic matter, between 60 and 71 % for crude protein and between 59 and 74 % for neutral-detergent fibre. Theoretical degradability was lower in summer than in spring and especially in autumn ($P < 0.001$). Reducing N fertilizer application decreased the theoretical degradability in the rumen ($P < 0.001$). Differences between rates of N application were less pronounced in autumn. © Elsevier / Inra

in situ / degradation / rye grass / fertilization / season

Résumé — Effets de la saison et du niveau de fertilisation azotée sur la dégradation in situ du ray-grass anglais exploité en pâturage. Utilisant la technique des sachets Nylon, la dégradation in situ du *Lolium perenne* a été étudiée sur des échantillons d'herbe pâturée, fertilisée à deux niveaux d'azote (250 et 550 kg N ha^{-1} an^{-1}), collectés en mai, juillet-août, septembre et octobre. La dégradabilité théorique dans le rumen a varié de 56 à 65 % pour la matière organique, de 60 à 71 % pour les matières azotées, et de 59 à 74 % pour le *neutral detergent fibre*. La dégradabilité théorique s'est révélée

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moins importante en été qu'au printemps et surtout qu'en automne ($p < 0,001$). La réduction de la fertilisation azotée a entraîné une diminution de la dégradabilité théorique dans le rumen ($p < 0,001$), mais les écarts entre niveaux de fertilisation se sont avérés moins importants en fin de saison.

in situ / dégradation / ray-grass / fertilisation / saison

1. INTRODUCTION

High rates of N application on grassland result in grass with high concentrations of readily degradable crude protein (CP) [26]. This results in excessive losses of N in the rumen of grass fed animals [27]. Excess N is excreted as urea N in urine, leading to volatilisation of ammonia and nitrous oxide and to nitrate leaching [10]. Changing grassland management in reducing N fertilizer input can diminish such losses. This seems necessary due to environmental regulations, particularly in intensive dairy husbandry areas [21]. The effect of N fertilization on in situ CP degradation has been investigated by several authors [7, 12, 14, 16, 20, 26, 29]. These studies were mostly restricted to primary growths of herbage under a cutting regime leaving the influence of N fertilization in late season and under a grazing regime unclear.

In the Dutch DVE/OEB protein evaluation system [22], rumen escape CP (E_{CP}) of grass is calculated by an equation including grass CP content and date of harvesting [25]. In this equation, E_{CP} increases linearly with advancing season and E_{CP} of autumn grass would therefore be higher than in summer. However, summer grass is characterized by a higher fibre content than grass in autumn or spring [1]. Because the proportion of E_{CP} increases with increasing fibre content [26], a quadratic relationship between E_{CP} and season would be expected.

The aim of this experiment was to study the effects of season on the in situ degradation of organic matter (OM), CP and neutral detergent fibre (NDF) of perennial ryegrass from grazed pastures, fertilized at two rates of N.

2. MATERIALS AND METHODS

2.1. Herbage samples

Herbage was collected in 1990 from pure swards of perennial rye grass (*Lolium perenne*) grown on clay soil at the Experimental Farm of Wageningen Agricultural University at Swifterbant, the Netherlands [3]. Plots were fertilized at either 250 or 550 kg N/ha/year, split into seven or eight dressings, respectively. Plots were grazed throughout the season for 1 or 2 days at a minimum yield of 1 500 kg dry matter (DM)/ha. Animal stocking rate was adjusted to an average daily DM allowance of approximately 16 kg per cow (i.e., 60 to 110 cows/ha). Grass height averaged 17 cm before grazing and 8 cm after grazing.

Immediately before grazing, plots were sampled. Within each plot, grass from 8 strips of 10 m was harvested with a reciprocating motor mower with a 0.88-m wide cutter bar, leaving stubble of 5 cm [5]. From the amount harvested, subsamples (minimum 6 kg) were collected by hand and stored in sealed plastic bags at –20 °C. Based on date of harvesting, subsamples were selected for in situ incubation (*table I*).

2.2. In situ incubation

Samples were prepared as described by van Vuuren et al. [26]. Nylon bags (polyamide; 10 × 19 cm, pore size 41 μm, porosity 30 %; Nybolt, Switzerland) were filled with (1- to 2-cm) chopped thawed grass equivalent to 5 g DM. Bags were closed and stored at –20 °C until rumen incubations. Another subsample was freeze-dried and ground to pass a 1-mm screen for chemical analyses.

Incubations were carried out in four Holstein-Friesian cows, producing 12 to 25 kg milk/d, fitted with large rumen cannulas (10 cm internal diameter; Bar Diamond Inc., Idaho, USA). Cows were grazing on a perennial rye grass pasture and supplemented daily with 4 kg of concentrates.

Prior to incubation, bags were thawed at ambient temperature for 2 h. Bags were incubated for 3, 6, 9, 12, 18, 24, 36, 48, and 336 h. Incubations were carried out in duplicate with a 5-week interval (June and July, 1992).

After removal from the rumen, bags were rapidly rinsed with cold tap water, placed in ice and subsequently washed in a domestic washing machine (V360, Bosch, Germany), providing four rinses of 15 L. Two additional bags per feed were washed without rumen incubation. Bags were dried at 70 °C for 24 h and weighed. Residues were ground to pass a 1-mm screen and composited per feed, cow, period, and incubation time.

Feed samples and incubation residues were analyzed for DM, ash, N and NDF. Analyses were performed as reported previously [28].

2.3. Calculations and statistical analysis

The instantly degradable fractions of OM (W_{OM}) and CP (W_{CP}) were estimated as fractions disappearing from the bags during washing (washout fraction). The disappearances of OM, CP and NDF from the bags during rumen incubation were fitted by a first order model [17]. This model included an insoluble, potentially degradable fraction (D), degraded at a constant rate (k_d) and an undegradable fraction (U). The U fraction was estimated as the residue after 336 h of incubation. Because lag time was not significantly different from 0 h, all parameters were calculated by a model without lag time. Theoretical degradability of OM (TD_{OM}), CP (TD_{CP}) and NDF (TD_{NDF}) in the rumen was calculated assuming a fractional passage rate (k_p) of 0.04/h for OM and CP [25] and 0.02 for NDF [9]:

$$TD = W + D \times k_d / (k_d + k_p),$$

where $W_{NDF} = 0$.

Results were analyzed by analysis of variance using Genstat [6]. Except for the W fraction, the effects of season and rate of N application were calculated within the block-structure cow × period:

$$Y_{ijkl} = \mu + C_i + P_j + (C \times P)_{ij} + N_k + S_l + (N \times S)_{kl} + \varepsilon_{ijkl}$$

where Y_{ijkl} is the observed value; μ is mean; C_i is cow effect; P_j is period effect; $(C \times P)_{ij}$ is interaction between cow and period; N_k is effect of rate of N application; S_l is season effect; $(N \times S)_{kl}$ is interaction between rate of N application and season, and ε_{ijkl} is the residual error, assumed to be normally and independently distributed.

3. RESULTS

3.1. Chemical composition

Concentrations of DM ranged from 155 to 241 g/kg. Rate of N application affected DM concentrations only for cuts 2 and 5 (*table I*). A higher rate of N application increased OM content by 5 to 12 g/kg DM and CP content by 25 to 65 g/kg DM. The difference in CP between both rates became smaller as the growing season progressed. Concentrations of NDF ranged from 481 to 529 g/kg DM and were not significantly affected by rate of N application.

In summer (July/August), DM concentrations were higher than in spring and autumn. From spring to autumn CP concentrations increased with a maximum in September. Concentrations of NDF were higher in September than in summer and October.

3.2. In situ degradation

In general, both rate of N application and cutting date influenced the in situ degradation of OM (*table II*), CP (*table III*) and NDF (*table IV*). The higher rate of N application increased rate and extent of degradation. Rate and extent of degradation at the end of the growing season were higher than in spring and summer. At the end of the growing season the differences due to rate of N application became smaller.

Table I. General characteristics and chemical composition of the grass samples incubated in situ.

	Rate of nitrogen fertilization										
	250 kg/ha/year Month of sampling				550 kg/ha/year Month of sampling				Effect[1]		
	May	Aug	Sept	Oct	May	July	Sept	Oct	N	S	N × S
Regrowth No.	2	5	6	7	2	5	7	8			
Days of regrowth	17	42	19	33	17	25	19	33			
Days after April 1	51	151	171	206	51	122	171	206			
Grass height before grazing (cm)	22.7	13.5	15.6	14.1	23.4	14.4	15	15			
Yield (10^3 kg dry matter/ha)	4	1.8	1.7	1.5	4.1	2.5	1.5	2			
Dry matter (g/kg DM)	202	241	155	175	177	234	157	175	***	***	***
Organic matter (g/kg DM)	903	891	882	882	907	897	894	891	***	***	***
N × 6.25 (g/kg DM)	182	208	285	273	247	261	328	298	***	***	
Neutral-detergent fibre (g/kg DM)	490	494	510	481	517	496	529	484	ns	*	ns

[1] Treatment effect of rate of N application (N), season (S) and interaction (N × S); ns, not significant; * $P < 0.05$; *** $P < 0.001$.

Table II. Effect of rate of N application and cutting date on the in situ degradation of organic matter of perennial rye grass.

Degradation Parameters[1]	Rate of nitrogen fertilization: 250 kg/ha/year, Month of sampling				550 kg/ha/year, Month of sampling					Effect[3]		
	May	Aug	Sept	Oct	May	July	Sept	Oct	SED[2]	N	S	N × S
W_{OM}	0.167	0.132	0.121	0.157	0.151	0.154	0.140	0.154	0.020	ns	ns	ns
D_{OM}	0.745	0.747	0.807	0.772	0.773	0.748	0.792	0.776	0.009	ns	***	*
U_{OM}	0.088	0.121	0.072	0.071	0.076	0.098	0.068	0.070	0.004	***	***	***
k_{dOM}	0.050	0.048	0.069	0.065	0.058	0.060	0.077	0.065	0.002	***	***	***
TD_{OM}	0.559	0.517	0.610	0.613	0.585	0.580	0.637	0.612	0.007	***	***	***

[1] W, instantly degradable fraction; D, potentially degradable, insoluble fraction; U, undegradadable fraction; k_d, fractional rate of degradation of D (/h); TD, theoretical degradability in rumen (assuming a fractional passage rate of 0.045/h). [2] Standard error of differences for means of the interaction between rate of N application and season. [3] Treatment effect of rate of N application (N), season (S) and interaction (N × S); ns, not significant; *, $P < 0.05$; ***$P < 0.001$.

Table III. Effect of rate of N application and cutting date on the in situ degradation of crude crude protein of perennial rye grass.

Degradation Parameters[1]	Rate of nitrogen fertilization: 250 kg/ha/year, Month of sampling				550 kg/ha/year, Month of sampling					Effect[3]		
	May	Aug	Sept	Oct	May	July	Sept	Oct	SED[2]	N	S	N × S
W_{CP}	0.140	0.078	0.078	0.105	0.153	0.102	0.133	0.128	0.026	ns	ns	ns
D_{CP}	0.785	0.838	0.882	0.847	0.793	0.840	0.828	0.827	0.011	**	***	***
U_{CP}	0.075	0.084	0.046	0.048	0.054	0.058	0.039	0.045	0.004	***	***	***
k_{dCP}	0.081	0.075	0.098	0.089	0.098	0.085	0.102	0.089	0.003	***	***	***
TD_{CP}	0.643	0.599	0.674	0.666	0.695	0.652	0.707	0.675	0.006	***	***	***

[1] W, instantly degradable fraction; D, potentially degradable, insoluble fraction; U, undegradadable fraction; k_d, fractional rate of degradation of D (/h); TD, theoretical degradability in rumen (assuming a fractional passage rate of 0.045/h). [2] Standard error of differences for means of the interaction between rate of N application and season. [s] Treatment effect of rate of N application (N), season (S) and interaction (N × S); ns, not significant; ** $P < 0.01$; *** $P < 0.001$.

Table IV. Effect of rate of N application and cutting date on the in situ degradation of neutral-detergent fibre of perennial rye grass.

Degradation Parameters[1]	Rate of nitrogen fertilization 250 kg/ha/year Month of sampling				550 kg/ha/year Month of sampling					Effect[3]		
	May	Aug	Sept	Oct	May	July	Sept	Oct		N	S	N × S
D_{NDF}	0.908	0.870	0.937	0.935	0.924	0.898	0.938	0.938	0.003	***	***	***
U_{NDF}	0.092	0.130	0.063	0.065	0.076	0.102	0.062	0.062	0.003	***	***	***
k_{dNDF}	0.040	0.042	0.065	0.060	0.048	0.052	0.075	0.061	0.002	***	***	***
TD_{NDF}	0.602	0.587	0.718	0.702	0.649	0.649	0.739	0.705	0.005	***	***	***

[1] D, potentially degradable, insoluble fraction; U, undegradadable fraction; k_d, fractional rate of degradation of D (/h); TD, theoretical degradability in rumen (assuming a fractional passage rate of 0.02/h). [2] Standard error of differences for means of the interaction between rate of N application and season. [3] Treatment effect of rate of N application (N), season (S) and interaction (N × S); *** $P < 0.001$.

3.2.1. Organic matter

Twelve to 17 % of the OM was washed out of the nylon bags, whereas the U_{OM} ranged from 7 to 12 %. This resulted in a D_{OM} of 75 to 81 %. The D_{OM} fraction disappeared in situ at a rate of 4.8 to 7.7 %/h.

Rate of N application had a significant effect on U_{OM}, k_{dOM} and TD_{OM} ($P < 0.001$), whereas W_{OM} and D_{OM} were not significantly affected. The theoretical degradability of OM varied between 56 and 64 % and increased with increasing level of N fertilisation, except in October. The effect was due to a lower U_{OM} and a higher k_{dOM} at 550 kg N/ha versus 250 kg N/ha.

Season had a significant influence on all parameters, except W_{OM}. The greatest changes occurred between July-August and September. Theoretical degradability of OM was low in May and July-August. The lower TD_{OM} of low-fertilized grass in July-August was due to the combination of a high U_{OM} and a low k_{dOM}. The higher TD_{OM} observed in September and October for both rates of N application was due to an increase in D_{OM} and k_{dOM}.

Interactions between rate of N application and season were most pronounced for U_{OM} and TD_{OM} and not significant for W_{OM}. Differences between rates of N application were especially high in July-August, due to a difference in growing days (*table I*).

3.2.2. Crude protein

The W_{CP} varied between 8 and 10 %, whereas U_{CP} varied between 4 and 8 % (*table III*). Consequently, 79 to 88 % of CP was insoluble but potentially degradable. The k_{dCP} varied between 8 and 10 %/h. The TD_{CP} ranged from 60 to 71 %.

The higher rate of N application increased TD_{CP}, but the difference decreased between May and October. Similar to OM, the higher TD_{CP} at the highest N application rate was associated with a lower U_{CP} and a higher k_{dCP}. The soluble CP fraction was higher at the higher application rate, but differences were not statistically significant.

Season had a significant influence on all degradation parameters ($P < 0.001$), except W_{CP}. Theoretical degradability of CP followed a quadratic pattern ($P < 0.001$) with a minimum value in summer. In contrast to U_{OM}, the changes in U_{CP} between May and July-August were smaller, but k_{dCP} declined more significantly than k_{dOM}. Higher U_{CP} fractions were found in May and July-August as compared to September and October. Between September and October k_{dCP} decreased significantly.

Significant interactions between rate of N application and season were apparent for all degradation parameters ($P < 0.001$), except for W_{CP}. An increase in U_{CP} for low-fertilized grass and a decrease in k_{dCP} for high-fertilized grass explain the decline in TD_{CP} between May and July-August. Similarly to OM, differences between application rates declined as season progressed.

3.2.3. Neutral-detergent fibre

The degradable NDF fraction varied between 87 and 84 % (*table IV*), whereas the k_{dNDF} varied between 4 and 8 %/h. Consequently the TD_{NDF} varied between 59 and 74 %.

Rate of N application had a significant effect on all parameters ($P < 0.001$). Whereas U_{NDF} was higher for the lowest application rate, D_{NDF} and k_{dNDF} were lower at this application rate. As a result, TD_{NDF} for 250 kg N/ha/year was always lower than for 550 kg N/ha/year.

The effect of season on NDF was similar to that on OM. Theoretical degradability of NDF was highest in September and October for both rates of N application. In September and October D_{NDF} and k_{dNDF} were higher than in the previous periods.

As observed for OM and CP, differences in degradation parameters between rates of N fertilization diminished as season progressed.

4. DISCUSSION

4.1. Chemical composition

An increase in application rate of N fertilizer usually decreases the DM content and increases CP concentrations of grass [23, 34]. This was confirmed by our observations for the first two periods, but in autumn rate of N application had no influence on the DM content.

The effect of rate of N application on NDF is influenced by the cutting strategy. If grass is sampled at a similar DM yield, differences in rate of N application may result in differences in stage of maturity. Usually maturity has a greater influence on chemical composition and nutritive value of grasses, than rate of N application [23]. In our study, grass of both application rates were sampled at the same age, except in summer when grass fertilized at 250 kg N/ha/year was 17 days older then grass at 550 kg N/ha/year. Despite the difference in growing days in summer, the difference in NDF concentrations was low. Concentrations of NDF were similar or slightly higher for high-fertilized grass. This agrees with observations of Valk et al. [23]. As demonstrated by Valk et al. [23], the higher NDF content is mainly due to an increase in neutral-detergent insoluble protein. When these authors corrected NDF concentrations for neutral-detergent insoluble CP, the expected inverse relationship between rate of N application and fibre content became apparent.

4.2. In situ degradation

Sample preparation is a major factor for variance in degradation parameters between laboratories [33]. Our data agreed with observations reported by Beever et al. [2], van Vuuren et al. [26], Waters and Givens [31], Steg et al. [19] and Valk et al. [23]. In those studies, grass samples were chopped prior to in situ incubation, which resulted in relative low W fractions (0.10 to 0.25) and consequently relatively high D-fractions (0.60 to 0.70). These observations are in contrast with those made by van Vuuren et al. [29] and Le Goffe et al. [13]. In these latter studies, grass was lyophilized and ground prior to incubation, which resulted in relatively high W fractions (around 0.5) and lower D fractions. Le Goffe et al. [13] also reported higher fractional degradation rates than in other studies.

Prior to rumen in situ incubations, samples were frozen and thawed twice, which could also affect protein degradation. Ruptures in cuticle and cell walls will facilitate the access for micro-organisms thereby increasing substrate availability. However, MacRae et al. [35] observed a reduction in protein solubility due to freezing, which they attributed to protein precipitation at lower pH.

Theoretical degradability summarizes all aspects of the in situ degradation. Present values are in concordance with van Vuuren et al. [26, 29] for similar rates of N application. *Figure 1* represents the calculated TD_{CP} values (k_p = 0.045 /h) from literature data. The TD_{CP} values (0.82 to 0.90) obtained by Le Goffe and Vérité [11] were dramatically higher than the other data (0.57 to 0.77). Again difference in sample preparation is the most likely explanation for this contrariety. Le Goffe and Vérité [11] used lyophilized and ground *Lolium perenne*, whereas all other workers used frozen and chopped *Lolium perenne*, except for Beever et al. [2] who incubated fresh and chopped material. The observed TD_{CP} in our experiment is close, albeit slightly lower, to the 0.73 adopted in the PDI system [30].

Preparation of the residues after incubation is a major reason for variation in estimated protein degradability amongst laboratories [33]. Especially in high-fibre feeds, a substantial proportion of CP in the bags originates from micro-organisms [36], which will underestimate theoretical protein degradability. Kamoun (unpublished data) showed that bacterial protein induced an

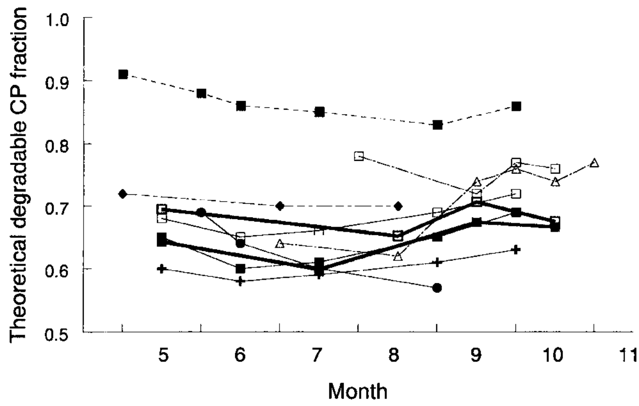

Figure 1. Influence of season on the theoretical degradability of crude protein in the rumen (assuming a passage rate of 4.5 %/h) of *Lolium perenne*. –◆– [2]; –·■–· [11]; –·△–·; –·□–· [31], two different sites; –●– [19]; –+–, –■–, –□– [23], 150, 300 and 450 kg N/ha, respectively; –■–, –□– with thick lines, current results, 250 and 550 kg N/ha, respectively.

underestimation of theoretical protein degradability varying from 10 to 20 %, depending on extent of cell wall lignification.

4.3. Effect of rate of N application

The effects of rate of N application on the U fraction and fractional degradation rate were similar to other studies [23, 26, 29]. Rate of N application had no consistent (OM) or significant (CP) effect on the W fractions, partly due to a high standard error. Similarly, Valk et al. [23] observed no significant effect of rate of N application on the W fraction. Probably, chopping with a paper guillotine resulted in a relatively high variation in sample composition amongst bags compared to freeze-drying and grinding. The tendency for a higher W_{CP} at a higher rate of N application can be explained by a higher amount of soluble, non-protein N [8] or by a smaller proportion of N bound to cell walls [32].

The greater theoretical degradability of OM, CP and NDF in the rumen of grass at the highest rate of N application, agrees with observations by Syrjälä-Qvist et al. [20] with timothy (*Phleum pratense*), Glenn et al. [7] with tall fescue (*Festuca arundinacea*) and cocksfoot (*Dactylis glomerata*), and Peyraud et al. [16] with *Lolium perenne*. In those studies, rates of N application were generally more modest than the present rates and studies most commonly applied to primary growths of herbage. With smooth bromegrass (*Bromus inermis*) hay, Messman et al. [15] demonstrated that N fertilization (89 kg N/ha versus nil) generally increased k_{dNDF} (from 5.4 to 6.6 %/h), whereas disappearance at 72 h was not affected. Le Goffe et al. [12] found no differences in TD_{CP} of tall fescue during the first three cuts, although rates of N application were high. However for the fourth cut, an increase in N application resulted in a higher TD_{CP}.

4.4. Effect of season

In our study, season had no effect on W_{OM}, whereas Beever et al. [2] and Steg et al. [19] observed a decline in W_{OM} as the growing season progressed. Season tended to have an effect on W_{CP}, which was lowest in July-August and highest in May. Beever et al. [2] observed similar trends in situ with fresh *Lolium perenne* although in their study W_{CP} was higher in late season. Abdalla et al. [1] reported that protein solubility (measured in a borate buffer) was highest in June and September and lowest in August for grazed herbage. These changes in N solubility may be related to changes in temperature, because protein solubility tended to be lowest when temperatures were the highest. In our experiment mean maximum temperatures in the week preceding sampling were 19 °C in May, 26 °C both in July and August, 17 °C in September and 15 °C in October.

The variations of grass composition and quality throughout the season result from the interaction of plant maturity and environmental influences, such as rainfall, temperature and light [23, 24, 26]. The highest k_d (September) may be justified by a difference in maturity stage (19 days in September versus 33 days in July-August and October). The low in situ degradation of the low-fertilized grass in July-August may be attributed to the high maturity (42 days of regrowth). More-mature grass is characterized by a lower availability of cell contents for microbial breakdown, resulting from increased cell wall lignification and increased proportion of N associated with cell walls [18].

Season had a quadratic effect on TD_{CP} ($P < 0.001$). From spring to summer TD_{CP} decreased, whereas from summer to autumn TD_{CP} increased again. The low TD_{CP} of the 5th cut was mainly based on the reductions in W_{CP} and k_{dCP} in July and August. This quadratic effect of season on TD_{CP} has been observed in other studies (*figure 1*). In the equations used in the DVE/OEB protein evaluation system to predict TD_{CP} and escape CP [4], season has a linear effect. This will result in an overestimation of the proportion of escape protein from grass at the end of the growing season. However, the predictions based on the equations of the DVE/OEB system underestimated the values based on our in situ data (*figure 2*). Also Valk et al. [23] suggested that the equations used presently [4] underestimate the proportion of escape protein. In agreement with our hypothesis, the magnitude of this underestimation decreased at the end of the growing season. These observations suggest that the equations to predict protein quality of rye grass require revision. Probably, the equations used presently are based on data from experiments in which grass samples were not representative for grass grazed under practical conditions.

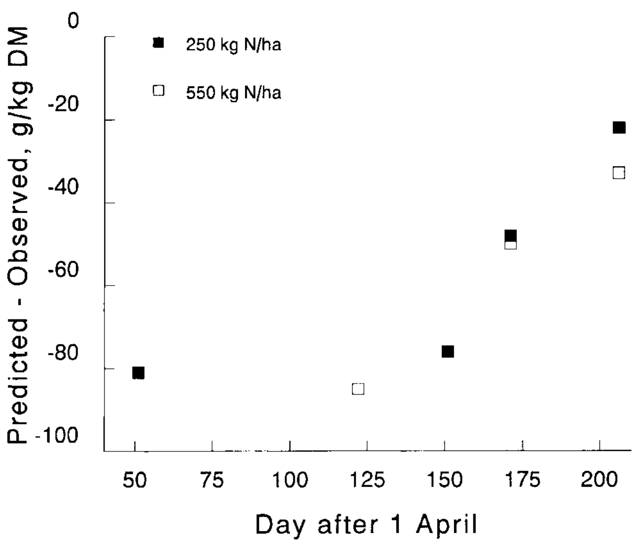

Figure 2. The effect of season on the difference between the proportion of escape feed protein predicted from equations in the DVE/OEB protein evaluation system and that estimated from data from the present experiment observed.

5. CONCLUSION

This study showed that the effect of season on the proportion of protein in perennial ryegrass that will escape from rumen fermentation is not linear as assumed by the prediction equations presently used [4]. Moreover, these equations led to an underestimation of the proportion of escape grass protein. With the expanded database on in situ degradation, improved equations to predict rumen degradability of grass protein should be derived.

ACKNOWLEDGMENTS

The authors are grateful to V.A. Hindle for his help during in situ incubations. The authors also wish to thank D.W. Bussink of the Nutrient Management Institute for providing the grass samples.

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