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Original Research Article

Sheep numbers required for dry matter digestibility evaluations when fed fresh perennial ryegrass or forage rape

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ABSTRACT

Research trials with fresh forages often require accurate and precise measurement of digestibility and variation in digestion between individuals, and the duration of measurement periods needs to be established to ensure reliable data are obtained. The variation is likely to be greater when freshly harvested feeds are given, such as perennial ryegrass (*Lolium perenne* L.) and forage rape (*Brassica napus* L.), because the nutrient composition changes over time and in response to weather conditions. Daily feed intake and faeces output data from a digestibility trial with these forages were used to calculate the effects of differing lengths of the measurement period and differing numbers of sheep, on the precision of digestibility, with a view towards development of a protocol. Sixteen lambs aged 8 months and weighing 33 kg at the commencement of the trial were fed either perennial ryegrass or forage rape (8/treatment group) over 2 periods with 35 d between measurements. They had been acclimatised to the diets, having grazed them for 42 d prior to 11 days of indoor measurements. The sheep numbers required for a digestibility trial with different combinations of acclimatisation and measurement period lengths were subsequently calculated for 3 levels of imposed precision upon the estimate of mean dry matter (DM) digestibility. It is recommended that if the standard error of the mean for digestibility is equal to or higher than 5 g/kg DM, and if sheep are already used to a fresh perennial ryegrass or forage rape diet, then a minimum of 6 animals are needed and 4 acclimatisation days being fed individually in metabolic crates followed by 7 days of measurement.

1. Introduction

Apparent total tract digestibility (feed digestibility) is commonly used to indicate the nutritive value of a feed. It reflects the availability of nutrients to the animal, by estimating the proportion of feed that is not excreted in the faeces but is assumed to be digested nutrients being absorbed from the digestive tract (McDonald et al., 2011). A very small proportion of feed dry matter (DM) is lost to carbon dioxide and methane, but this may be ignored for our purposes. The conventional method to determine digestibility is *in vivo* total faecal collection technique by recording the amount of feed eaten and faeces excreted. Other approaches, such as *in vitro* and *in sacco* techniques and chemical and physical measurements, offer a quicker and cheaper alternative (Kitessa et al., 1999; Minson, 1990), but these approaches rely on *in vivo* measurement for validation. Therefore, the total faecal collection technique is the standard method and still widely used.

The measurement of digestibility in feed evaluation and animal nutrition studies is important, yet researchers have been aware for more than 100 years of errors associated with digestibility measurements. For example, Grindley et al. (1917), cited by Schneider and Flatt (1975), recommended that "not less than three animals

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should be used in each lot". They also suggested if feasible five or even more animals should be used and commented that results obtained with more than 4 animals are much more reliable than those obtained with 1 or 2. The number of digestibility trials has grown over the years, and protocols for measuring digestibility have developed, incorporating such factors as animal choice, equipment, experimental procedure which affect digestibility. These have been summarised and reviewed (Cochran and Galyean, 1994; Grassland-Research-Institute, 1961; McDonald et al., 2011; Minson, 1990; Schneider and Flatt, 1975). Most of these recommended protocols were based on experience rather than experimental data, and sometimes recommendations were contradictory. However, some studies did provide experimental data for comparing digestibility protocols (Forbes et al., 1946; Raymond et al., 1953).

Animal husbandry in New Zealand relies on animals eating fresh forage. Fresh forage differs from dry or other conserved diets in many respects. Dry matter content varies from day to day, and nutritive value varies because the chemical composition of pasture changes continuously over time – between and within days. An animal's individual preference for and selection of particular forage species and plant parts further increases the variation in material eaten and nutrient intake (McDonald et al., 2011). Previous attempts to reduce feed intake variation have included harvesting and drying or freezing (Grassland-Research-Institute, 1961; Heaney et al., 1969), however, this approach might not be appropriate, either because drying or freezing facilities are unavailable or because it is imperative to conduct experiments with fresh forages where objectives relate to grazing. In addition, drying or freezing causes physical changes that result in quite different plant characteristics to pasture. For these reasons daily cut-and-carry protocols are required.

Perennial ryegrass (*Lolium perenne* L.) is the predominant forage species in New Zealand. Forage rape (*Brassica napus* L.) has both a high yield and nutritional value and is increasingly used by farmers (Barry, 2013). Forage rape differs from grass because it is a dicotyledonous plant with morphological and chemical differences, and may contain compounds that are deleterious to ruminants (Barry, 2013). Protocols which are designed for evaluating and measuring digestibility of grasses may be less applicable when applied to forage rape. Accurate determination of digestibility, as well as of its variation between and within individual animals, is required to enable proper comparison between cultivars, and also to develop industry recommendations for their use.

Many trials involving measures of digestibility include 2 phases: 1) A few days' acclimatisation or adaptation to the diet and facilities followed immediately by, 2) A number of days of measurement of feed eaten and faeces excreted. The feeding and measurements in these 2 phases, together with the number of animals used and the duration of each phase, are key factors associated with errors in digestibility measurements, but they could be standardised to develop a digestibility protocol for sheep (Schneider and Flatt, 1975). The objective of this study was to determine the minimum number of days of these 2 phases, and the number of animals required to estimate digestibility for fresh perennial ryegrass and forage rape.

2. Materials and methods

2.1. Animals, housing and experimental design

The management of animals described here was approved (No. 12645) by the Grasslands Animal Ethics Committee (AgResearch Ltd., Palmerston North, New Zealand). This study was conducted near Palmerston North, New Zealand, from May to September 2012 with 8-month-old Romney cryptorchid lambs: 112 individuals having a mean live weight of 33 ± 2.3 kg (mean ± SD). The lambs were randomly allocated to a diet of either perennial ryegrass (*L. perenne* L. variety *Ceres One* 50 containing endophyte AR1) or forage rape (*B. napus* L. variety *Titan*), with 56 sheep in each group. The forage rape diet was introduced gradually over 1 week, and the lambs were grazed on it for another 5 weeks prior to measuring digestibility indoors. Over the same time interval, the lambs in the ryegrass group were grazed on pasture. Eight animals from each forage treatment were selected for the indoor trial; all were selected to have similar live weight (44 ± 2.2 kg).

The indoor measurements comprised total faecal collection over 2 experimental periods of 11 days each (9–22 July, and 27 August – 9 September, 2012). Between these 2 periods, the animals were grazed on their respective pastures for 35 days.

During each indoor period the sheep were held in pens (8/pen) for 3 days acclimatisation, and then transferred to metabolic crates where harnesses were attached for total faecal collections over a 10-day period. Fresh forages were provided twice a day and there was free access to water. Forages were harvested daily between 09:00 and 12:00 and stored at 4 °C prior to feeding at 16:30 and 09:30. An allowance of cut forage was given to all sheep, with a target amount of approximately 2.3 times the metabolisable energy (ME) requirement for maintenance (MEm), calculated at the beginning of the experiment. The actual amount provided depended on the dry matter content, which was 14.5% and 15.9% for ryegrass and 11.0% and 12.2% for forage rape in the first and second indoor feeding periods, respectively. Maintenance requirements were based on the Australian-Agricultural-Council (1990) feeding standards, with ME of forage DM predicted by infrared reflectance spectroscopy (NIRS; Bruker Optics, model MPA, Ettlingen, Germany) and described by Sun et al. (2010).

2.2. Sample collection and processing

Faeces were collected from faecal bags attached to the harnesses at 08:00 each morning, the fresh weight of faeces recorded, and 10% of the total faecal output subsampled for each sheep each day and stored at −20 °C. The subsamples were freeze-dried, followed by oven drying at 65 °C to a constant weight for DM estimation.

Four sub-samples of approximately 200 g were taken from each fresh forage every day during the two experimental periods. One of the four daily sub-samples was dried at 65 °C for 48 h and these were then pooled for each forage over each experimental period and the pooled sample sent for nutrient profile analysis by the Nutrition Laboratory of Massey University (Palmerston North, New Zealand), as described by Sun et al. (2012). The remaining three daily sub-samples were individually dried at 105 °C for 24 h to determine DM content of the forage (AOAC, 1990; method 930.15). The dietary chemical composition is presented in Table 1. Feed refusals collected at 08:00 were weighed each day, subsampled and dried at 65 °C for 48 h to estimate forage DM not eaten.

2.3. Sample size calculations

The aim of this trial was to obtain measurements of digestibility for all possible lengths of consecutive acclimatisation and measurement phase during the two 11-day indoor periods, in order to determine the standard deviations associated with various durations of measurement and to calculate the numbers of sheep required for treatment comparisons. The digestibility for a measurement phase of more than 1 consecutive day was calculated from the sum of DM intakes and faecal DM outputs.

The required sample size (i.e., the number of sheep) for any given length of measurement phase was calculated using the following equation:

Table 1
Chemical composition (g/kg DM unless otherwise noticed) of perennial ryegrass and forage rape during 2 total collection periods.[1]

Item	Perennial ryegrass[2]		Forage rape[2]	
	Period 1	Period 2	Period 1	Period 2
DM, g/kg	138	161	128	122
Organic matter	889	885	910	909
Crude protein	236	195	175	179
Lipid	37	33	29	26
Neutral detergent fibre (NDF)	475	503	161	175
Acid detergent fibre (ADF)	220	249	116	124
Lignin	33	31	33	53

[1] Values are from wet chemistry methods.
[2] Period 1, d 47 to 56; Period 2, d 96 to 105 of the experiment.

$$\text{sample size} = \left(\frac{\text{SD}}{\text{SEM}}\right)^2,$$

where SD is the standard deviation of digestibility (g/kg DM) between animals, and SEM is the standard error of the mean of digestibility. The values of SEM were set at 2.5, 5 and 10 g/kg DM; levels of precision imposed upon the estimate of mean digestibility. These calculations were conducted separately for ryegrass and forage rape in both periods.

3. Results

3.1. Forage DM, feed intake and faecal output

The forage DM content (g/kg) of ryegrass ranged from 113 to 174 during measurement period 1, and from 110 to 190 during period 2. With forage rape, the range was 109 to 164 during period 1 and 101 to 151 during period 2. This variation resulted in different amounts of DM offered (kg/d) each day, since the amount of feed offered was based on fresh weight. The range of DM offered was 0.99 to 1.61 kg/d and 1.57 to 2.09 kg/d for ryegrass, and 1.17 to 1.53 kg/d and 1.51 to 1.88 kg/d for forage rape in periods 1 and 2, respectively. Variation (SD value) in DM intake was higher for sheep fed ryegrass compared with sheep fed rape, in both measurement periods. The actual range in plane of nutrition ingested by sheep was 1.03 to 2.52 and 0.78 to 2.03 × MEm for ryegrass and 1.40 to 2.37 and 1.42 to 2.26 × MEm for forage rape in periods 1 and 2, respectively.

3.2. Number of animals, days of sample collection and acclimatisation days

The number of animals required for different lengths of acclimatisation and measurement phase was calculated using the Equation shown in Section 2.3. For example, for ryegrass in period

Table 2
The minimum number of animals required to achieve a SEM of no more than 5 g/kg DM when fed fresh forage near *ad libitum* for a digestibility trial, given differing numbers of acclimatisation and measurement days.

Diet	Period	The number of acclimatisation days in crates	The number of measurement days									
			10	9	8	7	6	5	4	3	2	1
Perennial ryegrass	1	1	4	3	2	5	3	4	7	8	12	20
		2		4	4	3	8	6	9	21	27	18
		3			6	6	4	13	9	18	60	143
		4				4	3	2	4	2	5	34
		5					4	3	2	5	4	11
		6						6	5	3	15	12
		7							8	6	4	66
		8								9	6	25
		9									18	19
		10										32
	2	1	7	10	12	12	16	23	13	27	65	74
		2		5	7	8	12	20	32	20	51	174
		3			4	7	8	15	25	67	45	89
		4				6	10	13	24	37	126	205
		5					8	13	18	22	41	275
		6						8	9	12	14	34
		7							14	20	40	46
		8								16	25	87
		9									8	10
		10										21
Forage rape	1	1	4	3	3	3	4	5	4	8	11	24
		2		4	3	3	3	5	6	5	8	12
		3			6	5	5	4	7	9	9	12
		4				6	6	6	6	10	19	89
		5					6	5	4	3	8	19
		6						6	6	3	2	4
		7							10	10	6	11
		8								12	14	12
		9									17	26
		10										20
	2	1	3	4	4	4	4	6	5	8	13	11
		2		3	4	4	4	4	7	4	11	21
		3			3	3	4	4	3	5	2	16
		4				2	3	4	3	2	7	10
		5					3	4	5	6	5	21
		6						2	3	3	7	11
		7							3	5	6	10
		8								4	8	16
		9									4	11
		10										7

2, using a combination of 2 acclimatisation days and 6 measurement days, DM digestibility values calculated for the 8 animals were 801, 801, 785, 794, 796, 798, 758 and 796 g/kg DM, respectively. These values resulted in an SD of 14.3 g/kg DM. Setting a SEM of 5 g/kg DM, the resulting number of animals required for a digestibility trial is calculated to be 8.

The sample sizes were calculated based on SEMs set at 5 g/kg DM (Table 2), 10 g/kg DM (Table 3) and 2.5 g/kg DM (Table 4). The calculated number of animals required depended on the SD of digestibility over all 8 animals and tended to decrease with an increase in the number of measurement days and/or an increase in the number of acclimatisation days. The calculated numbers of animals differed between the 2 measurement periods, which might be due to the changes in diet chemical composition and resulted in large variation in the amount of refusals.

The minimum required number of animals with a SEM = 10 g/kg DM (Table 3) was generally equal to or less than 4 for combinations of more than 3 measurement days and any number of acclimatisation days.

The number of animals required for a digestibility study with SEM = 5 g/kg DM (Table 2) was higher than when the SEM = 10 g/kg DM (Table 3). Six animals or fewer were required for most combinations having in excess of 7 measurement days and any number of acclimatisation days.

The minimum number of animals needed for a digestibility trial with ryegrass or forage rape where SEM = 2.5 g/kg DM had a wide range from 5 to 1,097, and exceeded 7 animals per treatment for almost all the combinations (Table 4).

4. Discussion

4.1. Acclimatisation days

The purposes of the acclimatisation phase are 1) to adjust the animals to a new environment and feed so as to allow the residues of previous feeds to be expelled from the digestive tract and 2) to establish a steady rate of passage of feed through the digestive tract (Grassland-Research-Institute, 1961) allowing the feed intake to be calculated. An acclimatisation phase of 7 days was recommended by Grassland-Research-Institute (1961), and 6 to 8 days by Heaney et al. (1969). But if the feed type or feeding level is to be dramatically altered, the acclimatisation phase is suggested to be longer (Grassland-Research-Institute, 1961; Heaney et al., 1969). Our unpublished results in other studies show that it needs 7 days to completely expel Cr-mordanted fibre from the digestive tract. An acclimatisation phase of 7 days can only fulfil the first purpose. In our study, we had long durations of acclimatisation to the new diet of 42 and 35 days in the paddock, which was more than enough for this purpose.

Table 3
The minimum number of animals required to achieve a SEM of no more than 10 g/kg DM when fed fresh forage near *ad libitum* for a digestibility trial, given differing numbers of acclimatisation and measurement days.

Diet	Period	The number of acclimatisation days in crates	The number of measurement days									
			10	9	8	7	6	5	4	3	2	1
Perennial ryegrass	1	1	1	1	1	2	1	1	2	2	3	5
		2		1	1	2	3	2	3	6	7	5
		3			2	2	1	4	3	5	15	36
		4				1	1	1	1	1	2	9
		5					1	1	1	2	1	3
		6						2	2	1	4	3
		7							2	2	1	17
		8								3	2	7
		9									5	5
		10										8
	2	1	2	3	3	3	4	6	4	7	17	19
		2		2	2	2	3	5	8	5	13	44
		3			1	2	2	4	7	17	12	23
		4				2	3	4	6	10	32	52
		5					2	4	5	6	11	69
		6						2	3	3	4	9
		7							4	5	10	12
		8								4	7	22
		9									2	3
		10										6
Forage rape	1	1	1	1	1	1	1	2	1	2	3	6
		2		1	1	1	1	2	2	2	2	3
		3			2	2	2	1	2	3	3	3
		4				2	2	2	2	3	5	23
		5					2	2	1	1	2	5
		6						2	2	1	1	1
		7							3	3	2	3
		8								3	4	3
		9									5	7
		10										5
	2	1	1	1	1	1	1	2	2	2	4	3
		2		1	1	1	1	1	2	1	3	6
		3			1	1	1	1	1	2	1	4
		4				1	1	1	1	1	2	3
		5					1	1	2	2	2	6
		6						1	1	1	2	3
		7							1	2	2	3
		8								1	2	4
		9									1	3
		10										2

Table 4
The minimum number of animals required to achieve a SEM of no more than 2.5 g/kg DM when fed fresh forage near *ad libitum* for a digestibility trial, given differing numbers of acclimatisation and measurement days.

Diet	Period	The number of acclimatisation days in crates	The number of measurement days									
			10	9	8	7	6	5	4	3	2	1
Perennial ryegrass	1	1	13	12	8	20	10	14	27	30	46	77
		2		15	14	9	33	21	34	84	108	70
		3			23	21	15	49	34	70	238	572
		4				13	9	5	16	5	20	135
		5					16	11	7	18	15	42
		6						22	17	10	59	48
		7							30	24	16	261
		8								36	24	1,000
		9									69	73
		10										126
	2	1	28	46	48	63	89	52	108	258	293	
		2		25	32	45	78	126	78	201	695	
		3			25	31	58	98	266	180	354	
		4				21	37	51	95	146	503	818
		5					29	49	72	88	163	1,097
		6						30	35	48	54	134
		7							54	80	158	183
		8								61	98	345
		9									32	39
		10										83
Forage rape	1	1	14	11	11	9	16	19	16	32	41	94
		2		16	12	12	10	18	22	17	29	47
		3			23	20	18	16	25	35	35	47
		4				26	24	23	21	39	73	353
		5					24	20	15	12	30	73
		6						24	21	12	6	16
		7							37	38	23	43
		8								48	54	48
		9									68	104
		10										79
	2	1	11	14	15	15	14	22	18	31	51	41
		2		11	15	16	16	14	25	16	42	83
		3			9	12	13	14	11	19	7	62
		4				8	12	13	10	8	27	40
		5					10	15	17	23	20	82
		6						8	10	12	26	41
		7							9	18	24	40
		8								14	31	61
		9									16	41
		10										26

The indoor acclimatisation phase in our study was for the second purpose only. During this phase, animals should be fed an equal amount of DM at fixed times each day to ensure that a "steady state" of faecal excretion is reached and maintained during the collection phase (Blaxter et al., 1956). In our study, sheep were fed in pens for 3 days and in metabolic crates for 1 to 10 days for acclimatisation and fed the same amount of fresh weight each day. Daily intake varied due to the daily variation in DM content of fresh feed offered. The SD of the DM content of the forages in this study was 20.4 and 27.2 g/kg for ryegrass, and 17.2 and 12.9 g/kg for rape in indoor measurement periods 1 and 2, respectively. This is comparable to the results reported in other studies. For example, Tebot et al. (2012) reported the SD of pasture (90% oat [*Avena sativa*] and 10% white clover [*Trifolium repens*]) DM content was 17 g/kg for pasture cut in the early vegetative stage and 31 g/kg for pasture cut in the late vegetative stage of the harvest season. Despite the variations, the mean values of feed intake and faecal output in the first 3 to 4 days were close to the overall average (the difference less than 10%), suggesting 3 to 4 days of acclimatisation in individual crates are sufficient. Assuming that 7 days are needed for adaptation to diets (this assumption is based on our unpublished data that 7 days are needed to expel Cr-mordanted fibre from the digestive tract of sheep), together with 3 days in pens and 3 to 4 days in individual metabolic crates to achieve a steady state of faecal excretion, the total length of acclimatisation was 13 to 14 days. This length is similar to that recommended by McDonald et al. (2011) and what most researchers used (e.g., Fanchone et al., 2012; Archimède et al., 2000).

4.2. Measurement days

Grassland-Research-Institute (1961) suggested that the collection or measurement phase should be as long as possible, preferably of 10 to 12 days duration. Heaney et al. (1969) concluded that the collection phase is usually 4 to 12 days. These authors also advised that a period of less than 7 days should be avoided since the endpoint errors in faecal measurement decrease in direct proportion to the length of the collection phase since faecal output from the tested feed starts 1 or 2 days after the feed is eaten in ruminants (McDonald et al., 2011).

In our study, as the number of collection days increased up to 6 or 7 days, the numbers of animals required were greatly reduced, but lengths beyond that were not so useful in reducing animal numbers. Furthermore, having animals staying in metabolic crates with restricted movement for 10 to 12 days or more could be detrimental to animal welfare. We recommend that a collection period of 6 to 7 days should generally be sufficient.

4.3. Number of animals

Various numbers of animals for digestibility experiment are recommended in the literature. Raymond et al. (1953) and Grassland-

Research-Institute (1961) recommended 3 to 4 sheep. Heaney et al. (1969) concluded that 3 to 5 sheep are used per herbage in most digestibility experiments. Schneider and Flatt (1975) suggested that 4 to 6 animals per treatment are adequate and less than 3 animals should be avoided although it is preferable to have as many animals as possible. Cochran and Galyean (1994) recommended using 3 to 6 animals per treatment. When fed *ad libitum*, 8 to 10 sheep are required according to Minson (1990). The recent recommendations are that a minimum of 3 (Ajmal Khan et al., 2003) or 4 (McDonald et al., 2011) animals per treatment are required. Although ruminant species, feed type and confidence interval of digestibility were not always specified in these commendations, no more than 6 sheep are suggested unless fed *ad libitum*.

In general, the more animals that are used, the more accurate estimates can be obtained. However, this must be balanced against the fact that the total faecal collection procedure used to determine nutrient digestibility is time consuming, labour intensive and expensive. It is generally agreed that the number of animals should be kept to the minimum, but at the same time be large enough to adequately estimate the variation between animals, and other errors associated with digestibility measurements. The variation associated with digestibility estimates declines, especially for digestibility levels lower than 600 g/kg DM (Minson, 1990). This does not apply to the perennial ryegrass and forage rape used in this study since both forages had digestibility around 800 g/kg DM. Feed intake also affects the variation in digestibility (Minson, 1990). In the current study, sheep were offered 2.3 times the energy maintenance requirement, which was close to *ad libitum* feeding level. This feeding level would be expected to require more animals than lower feeding levels. Thus, the numbers of animals recommended by this study will be sufficient for other digestibility experiments on perennial ryegrass and forage rape as long as the feeding level is no more than in this study. Generally, with an increase in acclimatisation days or in measurement days, the minimum number of animals required is expected to decrease, and this trend was found in the present study (Table 2).

When we imposed the lowest precision upon the estimate of mean digestibility, that is SEM = 10 g/kg DM, fewer sheep were required. In the highest variance scenario (i.e., ryegrass in period 2), 4 sheep were required with a combination of 3 acclimatisation days and 5 measurement days. We think it is questionable whether a precision of SEM = 10 g/kg DM is sufficiently precise for a digestibility study although sometimes the SEM of DM digestibility between 10 and 13 g/kg DM is accepted (Raymond et al., 1953). We say this because a 95% confidence interval for the mean is from (mean − 1.96 SEM) to (mean + 1.96 SEM). Thus if the mean of digestibility is 800 g/kg DM, the 95% confidence interval will be from 780 to 820 g/kg DM.

When we imposed the highest precision upon the estimate of mean digestibility (i.e., SEM = 2.5 g/kg DM), it resulted in the requirement of the largest numbers of sheep. Twenty three sheep were needed for a combination of 3 acclimatisation days and 8 measurement days, and even more sheep for other combinations. This number of sheep in a total faecal collection trial is not feasible, and indeed such a high level of precision might be unnecessary for most total faecal collection trials. With the SEM set at 5 g/kg DM (i.e., a 95% confidence interval of digestibility is mean ± 10 g/kg DM), 6 sheep were required in the highest variance scenario (i.e., ryegrass in period 2) with 4 acclimatisation days and 7 measurement days. This level of precision of digestibility seems to us to be sufficient for most digestibility trials.

5. Conclusions

The minimum durations of acclimatisation in metabolic creates and measurement days, and the minimum required number of animals needed for a digestibility trial with fresh perennial ryegrass or forage rape are interdependent, and also depend on the desired precision. The acclimatisation and measurement phases should be as short as possible, but still guarantee that the animals are fully acclimatised to the diet, whilst at the same time providing a good degree of measurement precision. Based on the results of this study, it is recommended that the SEM of digestibility is set at 5 g/kg DM and, once sheep have already acclimatised to a fresh perennial ryegrass or forage rape diet, a minimum number of 6 animals, 4 acclimatisation days and 7 measurement days should be used for a digestibility study where the animals are fed individually in crates.

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