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SmartCow: an integrated infrastructure for increased research capability and innovation in the European cattle sector

an integrated infrastructure for increased research capability and innovation in the European cattle sector

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EXECUTIVE SUMMARY

Background	In vivo techniques relying on total collection of faeces and urine (total tract diet digestion, N balance) are considered a Gold Sandard Method (GSM) for phenotyping animals for feed efficiency (Martin, 1966; Spanghero and Kowalski, 1997), although the accuracy and precision of the results are compromised by technical limitations and there are concerns about staff, time, and safety, as well as animal welfare. Although the methods have been used for over 150 years, there is concern that aspects of their use at individual research facilities varies considerably, especially in terms of the number of days of collection used and the rigour in which collection of excreta and processing of samples is undertaken. One of the aims of SmartCow (https://www.smartcow.eu/) is to identify sources of variation in key GSM *in vivo* measurements of feed efficiency, including diet digestion and N excretion and balance. This will enable application of the 3-Rs by reducing variability, increasing precision and accuracy, and standardizing the methods and procedures used across research facilities within SmartCow installations and more widely. SmartCow WP5, working alongside WP3 for standardization issues, will evaluate and enhance the techniques being used, as well as contribute to the development of alternative approaches within WP6 through sample provision. **As measurement of diet digestion is required for measurements of diet N utilization and balance, and measurements of faecal N excretion incorporate measurement of faecal dry matter excretion and digestion, it is difficult to separate measurements of 'digestion' and 'N balance' in the evaluation of historical data and techniques. For this reason, there is some unavoidable overlap in the deliverable reports 5.1 (optimized digestion trial protocols) and 5.2 (optimized N balance procedures).** **However, we differentiate our final recommendations between both documents, focussing on digestion protocols in D5.1 and more specifically on N balance procedures in D5.2.**
Objectives	The overall aim is to identify and address sources of variation in key in vivo measurements of dietary nutrient use efficiency and associated emissions of nitrogen by cattle, thereby improving the measurements and unifying the approaches used across SmartCow installations, providing a global standard. The objectives are to (i) improve the accuracy and precision of measurements, and (ii) unify the methods used across SmartCow infrastructures. Specific objectives are to develop optimised procedures for measurements of diet digestion and N balance using total collection of faeces and urine that are acceptable in terms of precision, accuracy, animal welfare, and resource efficiency.

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Methods	We used 3 approaches: **Meta-analysis:** A meta-analysis of existing measurements of diet digestion and N balance for lactating dairy and growing beef cattle was conducted to determine the extent to which differences in measurement techniques across locations (e.g. collection methods, sample handling, days of sampling, etc.) introduces variation in measurements. Briefly, a database containing 2835 individual cow measurements of diet digestion and full N balance from digestion trials carried out at 12 research sites was assembled that include data from SmartCow partners Individual cow measurements included dry matter intake (DMI), chemical composition of the diets fed, nutrient intakes, faecal DM and N outputs, urinary N excretion, milk yield and composition, and body weight and other animal characteristics (e.g. breed, physiological state, sex). Multivariable analysis was used to account for variation due to experiment, diet intake, diet composition, and animal characteristics in order to ascertain the extent to which location and methodology accounted for variation in measurements of faecal, urine and milk excretions of N and resulting N balance. **Equipment development:** Equipment and techniques for collection and sampling of faeces and urine were improved and further developed as needed at individual SmartCow installations to reduce variability, improve precision, and improve animal health and welfare during measurements. This included design and development of new digestion stalls at Uread and INRAE. See D5.1 for the detailed results. **Assessments of sources of variation:** Sources of variation in N digestion, excretion and balance measurements used at Uread, INRAE, WU, FBN and AU were assessed according to the methods being used. Daily measurements of diet dry matter and N digestion and urine N excretion were obtained over the course of 4 to 10 day digestion trials conducted at Uread (lactating dairy cows) and INRAE (growing cattle) to determine the impact of number of days of excreta collection on measurements of diet digestion and N excretion and balance. The data were also used to calculate the impact of days of collection on variation of the measurements and thus the number of animals required for measuring detectable differences. At Uread effects of freezing and thawing of samples and a faecal preservative were also assessed. Sources of variation in digestion and N balance measurements were also evaluated by adding additional measurements to ongoing trials conducted at AU, FBN and WU. At Uread and WU measurements of digestion and N balance were obtained whilst animals were housed in respiration chambers equipped for measurements of ammonia N losses. Additional samples of faeces, urine, milk, and blood were obtained from some of these trials for WP6 proxy measurements.
Results & implications	**The results of the digestion and N balance trial procedure evaluations (MS5.4) were reported and discussed in detail at a workshop held virtually on 5 January 2022.** Minutes from the meeting and individual presentations are available on the SmartCow collaborative website (https://www.smartcow.eu/) and available on request. These presentations and the minutes of discussions highlighted improvements made at each SmartCow installation and identified key sources of variation and areas of focus for future improvement in the precision and accuracy of measurements of digestion and N balance in cattle. The meta-analysis of N balance measurements found that for all data sets and variables, over half the variation in measured N excretion and N balance (retention) was explained by the effect of research site and individual experiment. Site accounted for 20 to 27 % of variation

in urine N excretion and N balance, and 37 % of milk N excretion. Variation due to research site was less for faecal N excretion (and digestion), but substantial variation due to experiment was observed (56 – 62 %), partly reflecting differences in experimental diets. There were no significant effects of specific methodology variables (e.g. days of collection or method of N analysis), thus the analysis suggests that variation across sites is due more to exactly how specific experimental methods are used, rather than the method used per se. In this regard, strict attention to detail in the conduct of methods and procedures and training of staff and students undertaking the experimental procedures at all levels, from feeding and collection of samples, through to sample storage, processing and analysis is critical for minimizing variation in measurements of N digestion, excretion and balance in cattle.

At both INRAE and Uread, new stalls for housing animals during digestion measurements were developed that sought to improve animal comfort and welfare, health and safety of staff caring for the animals and obtaining samples, and provide flexibility for use with different sizes and types of animals. Key features included the ability to adjust the stalls for different sizes of animals and to temporarily restrict animal movements (e.g. adjustable side panels) during milking or sampling. A notable feature of the new stalls at Uread was the installation of waterbeds for stall matts, which provided a striking improvement in cow comfort.

Key sources of variation in digestion and N balance trials addressed at individual locations included evaluation of effects of the number of days of sampling, volatile N losses during sample collection and processing, N analysis methods, and the impact of spot sample collection timing. The SmartCow consortium Publisso publication 'Methods in cattle physiology and behaviour research – Recommendations from the SmartCow consortium' includes a chapter on 'Nutrient Digestibility and Balance Studies' that describes recommended procedures for conducting digestion trials and N balance (DOI: 10.5680/mcpb007). **The results of the joint research activities for WP5 highlight key aspects of the procedures described in the book of methods and specific sources of variation that warrant further investigation, but do not suggest that major revisions of the current chapter are required.** Some of the key considerations highlighted for reducing variance and increasing the precision and accuracy of N balance measurements include:

General:

1. Strict attention to detail and protocols is critical to minimizing variation in the measurements obtained. Improvements can be achieved through routine self-assessment and critical evaluation at each installation. These evaluations should be conducted at each experimental facility as the application of specific methods and procedures will vary due to the historical use of procedures and modifications that have occurred over time.
2. Training of staff and students conducting trials and effective communication is essential to be sure all are fully engaged and committed to the success of the experiments and are aware of the implications of specific procedures and sources of variation.

Specific procedures:

1. Under conditions of measurement at INRAE, repeatability of measurements of digestion and N balance in growing bulls were greater after at least 7 days of collection and for digestibility was greatest after 10 days of collection.

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	2. Shorter periods of 4 days were acceptable for lactating dairy cows in a trial where intake and diet composition did not vary over the course of the measurement periods. 3. Cow comfort and adaptation and training to digestion stalls and collection equipment and procedures is important for reducing variability and minimizing stress. 4. Accurate measurement of diet intake and composition of feeds and refusals is critical. 5. It is essential that collection of faeces and urine is complete and no cross contamination occurs. Mixing of acid and urine throughout collection is highly recommended. 6. Frozen bulk samples of feed and faeces are acceptable for studies of N digestion and excretion if processed in a manner that minimizes volatile N losses. In this regard analysis of samples that have been subjected to minimal processing and kept chilled or frozen during chopping and mixing is essential. This can be achieved by mixing samples with dry-ice. Samples should not be dried before analysis unless volatile N losses can be accounted for. 7. Addition acid to faeces in these studies was not effective as a preservative and reduced N concentration of faeces and manure. 8. Urine should be analyzed fresh, if possible, otherwise the time spent frozen before analysis should be kept to a minimum. **Factors needing further investigation:** 1. Reasons for the apparent loss of N from frozen urine samples need to be determined and alternative approaches identified. 2. Reasons for the higher feed N concentration obtained using Dumas combustion compared to Kjeldahl at WU need to be identified and addressed. 3. Losses of ammonia during collection should be quantified, if possible, especially under conditions where there is mixing of urine and faeces during collection. In this regard procedures for ammonia emission measuring should be standardized and recovery tests conducted.

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1) Introduction

Whilst in vivo measurements of diet digestion and N excretion and balance have been used for over 150 years and are considered to be 'standard' techniques, there is concern that the methods used at individual research facilities vary considerably, especially in terms of the number of days of collection used and the rigour in which collection of excreta and processing of samples is undertaken. Once all routes of N intake and excretion are measured, N balance is calculated as the difference and is assumed to represent total body tissue retention or loss of nitrogenous compounds. As reviewed previously, these measurements of body N accretion can often be excessively high relative to biologically realistic rates of body protein accretion and measured weight gain (Martin, 1966; Spanghero and Kowalski, 1997 and 2001; Reynolds and Kristensen, 2008; Hristov et al., 2019). As any potential losses of N from feed, faeces, urine, milk, claws, or as hair and scurf that are not measured are included in the estimate of body N balance, it has long been hypothesized that the sum of these volatile N (e.g. ammonia) and other unmeasured N losses may contribute to the excessively high rates of body N retention often reported (Spanghero and Kowalski, 1997). Recent studies have shown the extent to which sample processing and analytical method can affect measured N concentrations of feed, faeces, urine and milk (Morris et al., 2019). In addition, there has been a trend in the literature for the length of digestion trials to be reduced, which may in part be occurring to minimize weekend labour requirements and minimize cost of the experiment. Historically digestion trials have been conducted over 7 days or more to account for daily variation in faecal excretion (Schneider and Flatt, 1975), and it is not certain if there is a minimal number of days of collection required for a sufficient reduction in variance to be reached in order to minimize the number of animals required for statistically meaningful results. This may in part depend on the production level of the animal and the type of diet fed, as it has previously been shown the CV for faecal dry matter excretion is higher for lactating cows than for dry cows (Schneider and Flatt, 1975).

Measurement of diet digestion is required for measurements of diet N utilization and balance, and measurements of faecal N excretion require measurement of faecal dry matter excretion and digestion, therefore it is difficult to separate measurements of 'digestion' and 'N balance' in the evaluation of historical data and techniques. For this reason, there is some overlap between the deliverable reports 5.1 (optimized digestion trial protocols) and 5.2 (optimized N balance procedures) in the report that follows.

2) Objectives:

The overall objective of task 5.1 was to optimise procedures for measurements of 1) diet digestion and 2) nitrogen (N) balance using total collection of faeces and urine that are acceptable in terms of precision, accuracy, animal welfare, and resource efficiency and are applicable for lactating dairy and growing beef cattle.

3) Methods:

3.1) Meta-analysis of existing data: First, historical measurements of diet digestion and N balance for lactating dairy and growing beef cattle were compared through a meta-analysis to determine the extent to which differences in measurement techniques across locations (e.g. collection methods, sample handling, days of sampling, etc.) introduces variation and bias in measurements. Potential sources of variation considered previously (e.g. Martin, 1966, Spanghero and Kowalski, 1997) were considered. Methodology used and specific details and results are described in the associated report (MS5.1 – appendix 1). Briefly, a database containing 3969 individual cow measurements of diet digestion and N excretion and balance from digestion trials carried out at 15 research sites associated with 11 different research institutions. The data base was assembled using data from SmartCow (https://www.smartcow.eu/) partners as well as the Feed and Nutrition Network of the Global Research Alliance (https://globalresearchalliance.org/research/livestock/networks/feed-nutrition-network/). Research sites were located in North America (3), Europe (11) and Oceania (1). The data set was comprised of 125 separate experiments with sites contributing data from 1 to 34 experiments (18 to 759 observations). Individual

cow measurements included dry matter intake (DMI), chemical composition of the diets fed, nutrient intakes, faecal DM and N outputs, urinary N excretion, milk yield and composition, and body weight and other animal characteristics (e.g. breed, physiological state, sex). Retained N balance was calculated by subtracting faecal N, urine N and milk N (if lactating animals) excretions from N intake. Complete N balance data was available for 12 sites (2835 observations) and only observations associated with complete N balance (as opposed to only N digestion) measurement were included in the meta-analysis, which was conducted much as described previously (van Lingen et al., 2019) to account for variation due to experiment, diet, intake, diet composition, and animal characteristics in order to ascertain the extent to which location and methodology accounted for variation in measurements of faecal, urine and milk excretions of N and resulting N balance.

3.2) Equipment development: Secondly, equipment and techniques for collection and sampling of faeces and urine were improved and further developed as needed at individual SmartCow installations to reduce variability, improve precision, and improve animal health and welfare during measurements. This included design and development of new digestion stalls at Uread and INRAE.

3.3) Assessments of sources of variation: Thirdly, daily measurements of diet dry matter and N digestion and urine N excretion were obtained over the course of 4 to 10-day digestion trials conducted at Uread (lactating dairy cows) and INRAE (growing beef cattle) to determine the impact of number of days of excreta collection on measurements of diet digestion and N excretion and balance. The data were also used to calculate the impact of days of collection on variation of the measurements and thus the number of animals required for detecting significant differences. Diets with low and excessive N concentration were used to produce a large difference in urine urea concentration and thus potential losses of ammonia from urine. At Uread effects of freezing and thawing of samples and a faecal preservative were also assessed. Sources of variation in digestion and N balance measurements were also evaluated by adding additional measurements to ongoing trials conducted at AU, FBN and WU. At Uread and WU measurements of digestion and N balance were obtained whilst animals were housed in respiration chambers equipped for measurements of ammonia emissions to quantify the extent of these volatile nitrogen losses. At WU, the impact of N measurement method (Dumas vs Kjeldahl), the addition of acid to manure as preservative, and losses of volatile N in the days after animals left chambers, were analysed. Additional 'spot' samples of faeces, urine, milk, and blood were be obtained from some of these trials for WP6 proxy measurements.

4) Expected outcomes:

Recommended measurement protocols that reduce variability of diet digestion measurement and minimise the number of animals and samples required from the animals used.

5) Key Results: Brief synopsis of the outcomes of the joint research activities for WP5:

The results of the digestion and N balance trial procedure evaluations (MS5.4) were reported and discussed in detail at a workshop held virtually on 5 January 2022. Minutes from the meeting and individual presentations are available on the SmartCow collaborative website or by request. These presentations and the minuted discussions highlighted improvements made at each SmartCow installation and identified key sources of variation and areas of focus for future improvement in the precision and accuracy of measurements of digestion and N balance in cattle.

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5.1) Meta-analysis of Individual N Balance Measurements: A full report of the methodology used, results, and discussion of the meta-analysis of variation in measurements of faecal (linked to digestion), urine, and milk N excretion and resulting N balance can be found in the full meta-analysis report (MS5.1; see below). Factors contributing to variation were assessed for measurements obtained in growing, nonlactating, and lactating cattle (n = 2817 or n = 1704) and lactating cattle only (n = 1277). Dietary and animal factors were addressed in order to better assess variation due to research site and methodology used. For all data sets and response variables over half the variation in measured N excretion and N balance (retention) was explained by the effect of the research site and individual experiment. Site accounted for from 20 to 27% of variation in urine N excretion and N balance, and 37% of milk N excretion (likely reflecting differences in milk yield of cows used at each site). Variation due to research site was less for faecal N excretion (and digestion), but substantial variation due to experiment was observed (56 – 62%, partly reflecting differences in experimental diets). Graphical representation of the variation in faecal N and N balance across research sites is shown in figures 1 and 2, respectively.

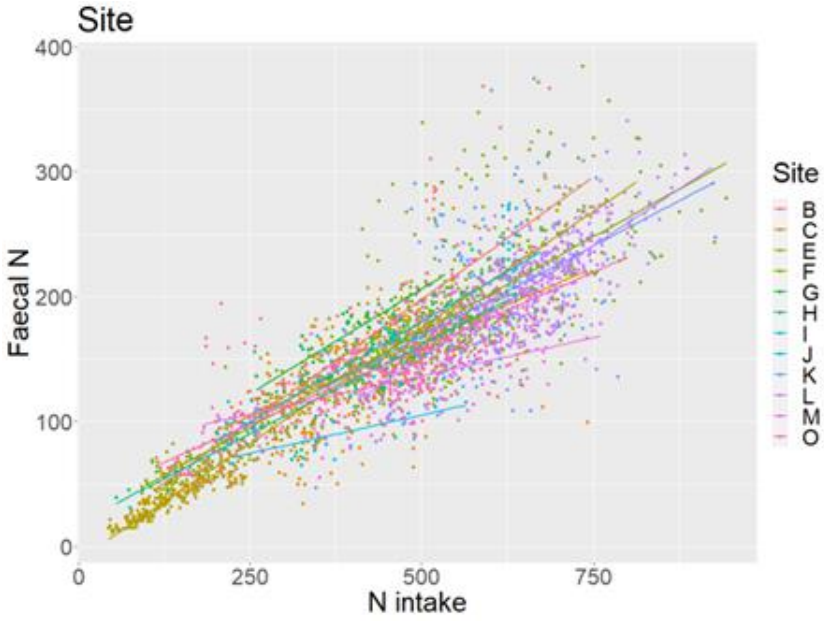

Figure 1. Relationship between faecal N excretion and N intake (g/d) for individual research sites.

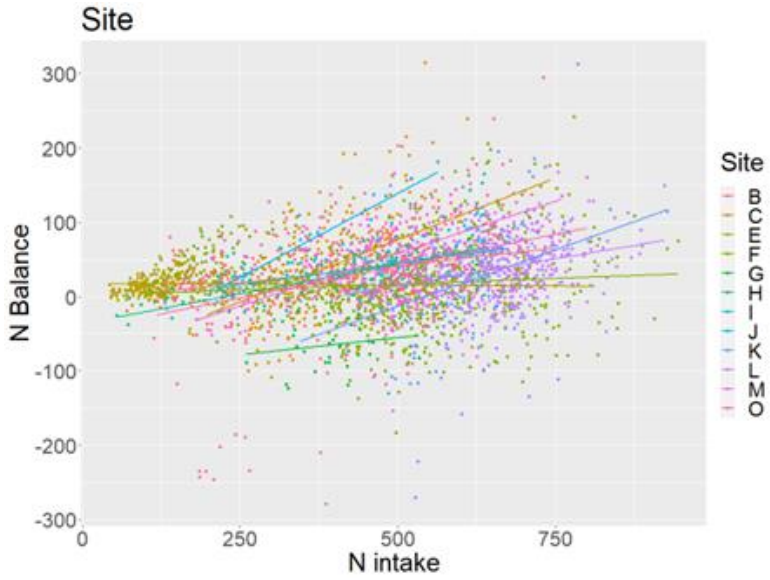

Figure 2. Relationship between N balance and N intake for individual research sites.

There were no significant effects of specific methodology variables (e.g. days of collection or method of N analysis), which suggests that in addition to differences in the production of the experimental animals studied, variation across sites may be due more to how the specific experimental methods are used, rather than the method used per se. In this regard strict attention to detail in the conduct of methods and procedures and training of staff and students undertaking the experimental procedures at all levels, from feeding and collection of samples, through to sample storage, processing and analysis is critical for minimizing variation in measurements of N digestion, excretion and balance in cattle (Snedecor and Flatt, 1970).

5.2) Equipment and procedure development (see D5.1 for the detailed results):

At both INRAE and Uread new stalls for housing animals during digestion measurements were developed that sought to improve animal comfort and welfare, health and safety of staff caring for the animals and obtaining samples, and provide flexibility for use with different sizes and types of animals.

Other improvements included adaptations to urine collection systems at Uread that minimized leaks and faecal contamination and provided constant mixing of urine and acid, which reduces N losses as ammonia. At WU respiration chambers were equipped for measurements of ammonia emissions to account for losses during manure collection.

5.3) Assessments of sources of variation:

As noted above, some of the key sources of variation in digestion and N balance trials addressed at individual locations included days of sampling, volatile N losses during sample collection and processing, N analysis methods, and the impact of spot sample collection timing.

a) Use of markers and spot sampling (AU)

At **AU** markers and spot-sampling of faeces is used as an alternative to total faecal collection and a trial was conducted to determine the most appropriate schedule of spot-sample collection. It was concluded that 3 samplings (morning, early afternoon, late afternoon) over 2 consecutive days was most appropriate based on the variation observed (previously 2 samples over 3 days was used).

b) Effect of sample drying and grinding (FBN)

At **FBN** the effect of sample drying and grinding on feed sample N concentration was determined. As shown in figure 5, N concentration of feed was 11 to 14% higher when frozen feed was ground with dry ice before analysis compared to drying at 60°C before grinding. FBN also observed that as much as 7 g of N is lost as gaseous ammonia when urine and faeces are not collected separately.

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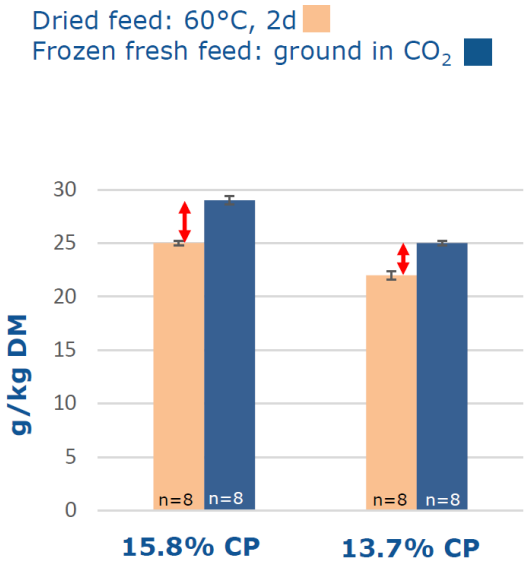

Figure 5. Effect of sample processing methods on N concentration of feeds with different crude protein (CP) concentrations.

c) N losses during measurements of N balance (WU)

WU conducted a number of assessments of N losses during measurements of N balance of lactating dairy cows within their climate-controlled respiration chambers. They found that method of feed N analysis had the largest impact on calculated N balance, with Dumas measurements higher than Kjeldahl. This is something that needs further assessment. WU collect urine and faeces together as manure, which results in ammonia losses from the chambers that can be measured and accounted for using acid traps and measuring ammonia in heat exchanger condensate. They also found that a gas detector instead of the acid traps was an appropriate alternative that required less labour. Adding acid to the manure as a preservative was found to increase ammonia losses rather than reducing them as is the case for urine on its own. They also found that some ammonia losses from the chambers continued to occur during cleaning of the chamber equipment and after the trial finishes, which should be measured and accounted for. As ammonia measurements are important for accurate measurements of N balance, it was decided that in future recovery tests should be conducted to determine the accuracy of measurements.

d) Assessment of the 'gold standard' measurement with 2 crude protein concentrations (UREAD)

Uread conducted a 4 x 3 switch-back design study using 4 mid-lactation Holstein cows fed ad libitum one of 2 total-mixed ration (TMR) diets differing in total CP concentration (14 vs 18%) and with 3 five-week periods. Cows were randomly assigned to one of the two diets (2 cows each) in the first period and then diets were switched at the beginning of each subsequent period (14-18-14 vs 18-14-18). Digestion and N balance was measured over 8-day periods using total collection of faeces and urine whilst cows were housed in respiration chambers equipped for measurements of ammonia in exhaust air and retained in air-conditioner condensate. Samples of feed, feed refusals, faeces, urine and milk were obtained daily and kept chilled until analyzed by macro-Kjeldahl analysis as soon as possible on the day of collection. Samples of feeds were coarsely chopped using dry-ice before analysis to minimize volatile N losses. Samples were not dried before analysis. The resulting measurements of N excretion and balance were considered to be as close as possible to a 'gold standard' measurement. Representative daily samples were also taken from 24-hour collections and added to a frozen bulk sample obtained over 4 to 8 days. These bulk samples were then frozen and analysed as soon as possible after completion of the trial (within 10 days). In period 2 only, additional bulk samples of faeces were obtained and treated with a solution of HCl-ethanol as a preservative and then either frozen or kept refrigerated until analysis during the week following the

completion of the trial to determine if a preservative reduced volatile N losses during thawing and freezing or would provide an alternative to freezing of samples.

A key feature of the planning for this trial was the involvement of all staff involved in weekly meetings to co-develop new equipment and standard operating procedures for digestion trials at Uread. Modifications were made to the new digestion stalls and urine and faecal collection equipment to minimize losses during collection and cross contamination and volatile N losses. This training and attention to detail were considered essential for precise and accurate measurements.

For feed and faeces samples, averages of daily N concentration made on fresh samples were similar to concentrations measured in frozen and thawed bulk samples, with no consistent trends in terms of frozen samples being higher or lower in N concentration. In contrast, urine samples that had been frozen were consistently lower in N concentration compared to samples analyzed on the day of collection. Concentrations were 7.17 vs 6.75 and 10.39 vs 9.97 g/kg for low and high CP diets, respectively. This has been reported previously (Morris et al., 2019) and requires further investigation. Morris et al. (2019) reported the decrease in frozen urine N concentration was greater with longer time in the freezer. When HCl-ethanol was added to bulk samples, N concentration was consistently lower compared to samples analyzed on the day of collection, suggesting that as observed in the WU trial acid addition to faeces increased volatile N losses during sample processing and analysis, rather than minimizing N losses as intended. Over the course of the 8 days of collection both feed intake and N concentration were relatively stable, with minimal variation in N intake and excretion when comparing data averaged over days 1-4 versus 1-8 (Tables 3 and 4). This may in part reflect both cow comfort as noted above and also training and acclimatisation of cows to the digestion stalls and chambers prior to the trials. When comparing data from 4-day and 8-day collections, there were some small differences in the absolute values for N intake, N excretion and treatment differences, particularly milk nitrogen which were compounded when nitrogen balance was calculated, however, the differences were not substantial (Tables 1 to 4). As expected, as sample size was increased from 4 to 8 days of collection the average standard errors associated with nitrogen intake and excretion were slightly (approximately 10%) lower when 8 days of collection were included in the analysis. This suggests that when animals are well adapted to digestion stalls and equipment and diet intake and composition is stable then measurements periods of 4 days would be acceptable for detecting significant differences across contrasting dietary treatments in terms of CP content. Similarly, N intake and excretion measured on the basis of frozen samples bulked over the first 4 days of the trial and analyzed for N concentration in the week following completion of the trial (Table 5) was comparable to measurements based on analysis of samples on the first 4 days of the trial (Table 4), although urine N concentration and excretion were slightly lower and N balance slightly higher. This suggests that frozen bulk samples are acceptable when handled carefully to minimize volatile N losses, although urine N concentration may be lower and thus N balance higher.

Table 1. Diet digestion and urine excretion based on an average of 8 daily samples

	Diet CP, %			P <1	
	14	18	SEM	Diet	Period
Dry matter intake, kg/d	19.53	20.98	0.958	0.134	0.614
Milk yield, kg/d	26.4	28.8	1.24	0.292	0.048
Faecal dry matter, kg/d	6.08	6.30	0.306	0.571	0.669
Urine, kg/d	18.47	22.94	0.747	0.001	0.019
Dry matter digested, kg/d	13.32	14.64	0.713	0.116	0.641
Dry matter digested, g/g	0.686	0.698	0.009	0.118	0.711

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Table 2. Diet digestion and urine excretion based on 4 day bulk sample analysis.

	Diet CP, %			P <	
	14	18	SEM	Diet	Period
Dry matter intake, kg/d	19.62	21.17	1.044	0.159	0.706
Faecal dry matter, kg/d	6.08	6.18	0.333	0.799	0.472
Urine, kg/d	18.46	22.75	0.921	0.003	0.033
Dry matter digested, kg/d	13.42	14.94	0.761	0.105	0.791
Dry matter digested, g/g	0.695	0.702	0.009	0.377	0.776

Table 3. N excretion and balance based on an average of 8 daily sample analyses.

	Diet CP, %			P <	
	14	18	SEM	Diet	Period
Nitrogen balance, g/d					
Intake	427	585	19.3	0.002	0.241
Diet CP, g/kg DM	136	172	0.77	0.001	0.002
Faecal	169	191	8.57	0.055	0.491
Faecal N, g/kg DM	27.7	30.4	0.323	0.001	0.201
Digested	258	393	16.3	0.003	0.2
Urine	133	238	10.54	0.001	0.003
Urine N, g/kg	7.2	10.4	0.401	0.001	0.052
Manure	302	429	17.95	0.001	0.06
Milk	135	160	7.46	0.139	0.302
Balance	-9.9	1.8	9.45	0.375	0.051
Condensate N, mg/d	374	420	69.1	0.713	0.780
Ammonia N, mg/d	132	473	234.2	0.385	0.225

Table 4. N excretion and balance based on an average of 4 daily sample analyses.

	Diet CP, %			P <	
	14	18	SEM	Diet	Period
Nitrogen balance, g/d					
Intake	421	583	20.6	0.003	0.372
Diet CP, g/kg DM	136	172	0.6	0.001	0.001
Faecal	166	188	9.3	0.114	0.376
Faecal N, g/kg DM	27.8	30.5	0.41	0.028	0.764
Digested	255	395	18.4	0.004	0.457
Urine	134	237	11.0	0.001	0.025
Urine N, g/kg	7.24	10.6	0.46	0.001	0.029
Manure	300	425	19.3	0.002	0.122
Milk	139	157	7.8	0.241	0.396
Balance	-14.9	6.2	12.8	0.225	0.205

Table 5. N excretion and balance based on 4-day bulk sample analysis.

	Diet CP, %			P <	
	14	18	SEM	Diet	Period
Nitrogen balance, g/d					
Intake	421	600	21.5	0.021	0.400
Diet CP, g/kg DM	134	174	2.1	0.011	0.080
Faecal	167	189	8.8	0.090	0.401
Faecal N, g/kg DM	27.5	30.5	0.40	0.001	0.780
Digested	253	410	17.5	0.001	0.120
Urine	128	232	11.0	0.001	0.031
Urine N, g/kg	6.9	10.2	0.48	0.001	0.070
Manure	295	421	19.0	0.002	0.104
Milk	135	153	8.5	0.280	0.500
Balance	-9.0	28.0	14.8	0.034	0.085

e) Evaluation of the effect of days of collection on the repeatability and precision of measurements (INRAE)

INRAE conducted an extensive evaluation of the effect of days of collection on the repeatability and precision of measurements of feed digestion and N excretion and balance of 16 growing bulls fed ad libitum diets differing in CP concentrations (17.3 vs 11.6 % CP, 8 bulls per diet) over the course of 2 digestion trials repeated at 9 week intervals. Bulls remained on the same dietary treatment through both digestion trials. The bulls were moved from free-stall housing to digestion stalls for 15 days, with 5 days of adaptation followed by 10 days of sample collection. Hair cortisol was also measured as an indicator of animal stress. The results showed that repeatability, measured as the within-diet correlation between measurements in the 2 periods, increased with the length of the collection periods for both feed dry matter digestibility and N balance . In addition, power tests were conducted to determine the effect of days of collection on the minimum detectable difference for digestibility and N balance. The results show that longer periods result in a lower detectable difference for dry matter digestibility (Figure 6), with the lowest detectable difference for 10 day collections (Deliverable 5.1). For N balance, a 20% difference in N balance could be detected with 16 animals per treatment and at least 7 days of collection (Figure 7). Measurements of hair cortisol were lowest when animals were housed in free stalls and increased during digestion trials, although concentrations were highest in the first days of collection, suggesting that animals adapted to the stalls over time.

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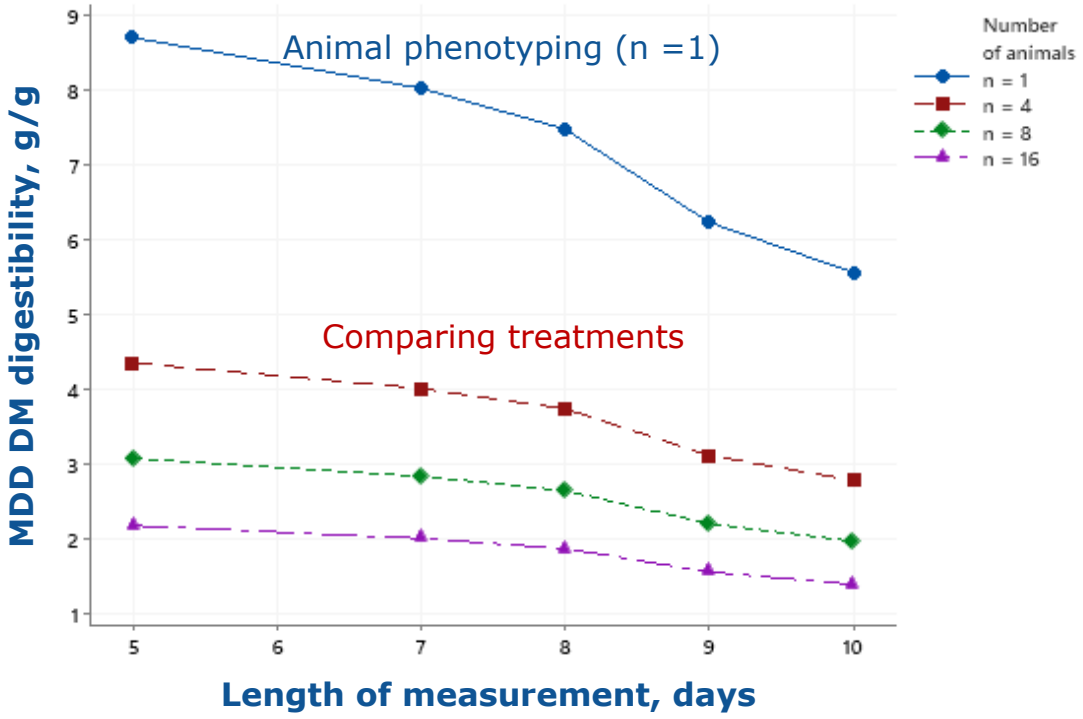

Figure 6. Minimum detectable difference (MDD, g/100g) and days of measurement of feed dry matter digestibility for 1, 4, 8 and 16 growing bulls.

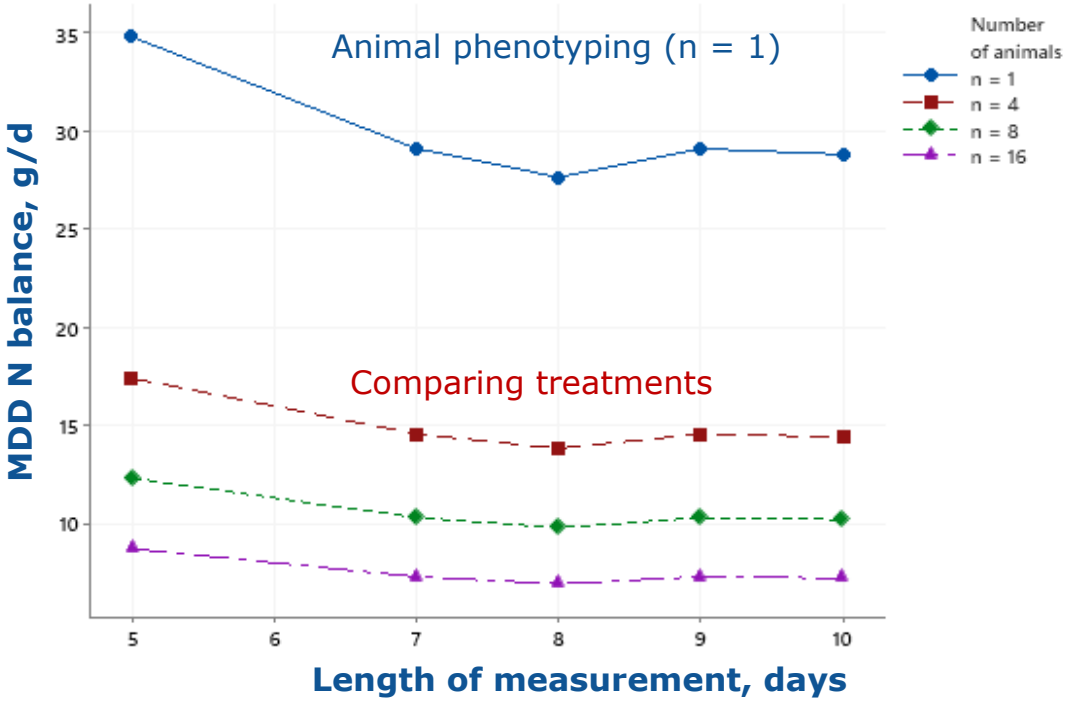

Figure 7. Minimum detectable difference (MDD, g/d) and days of measurement of N balance for 1, 4, 8 and 16 growing bulls.

6) Optimised digestion trial protocols (D5.1) and N balance procedures (D5.2).

Collective recommendations for best practice procedures for the conduct of nutrient digestibility and balance studies were published by the SmartCow consortium in the Publisso publication 'Methods in cattle physiology and behaviour research – Recommendations from the SmartCow consortium'. The chapter 'Nutrient Digestibility and Balance Studies' describes recommended procedures for conducting digestion trials and N balance.

Danesh Mesgaran S, Kuhla B, Baumont R, Cantalapiedra-Hijar G, Nozière P, Lund P, Humphries D, Dijkstra J. Nutrient digestibility and balance studies. In: Mesgaran SD, Baumont R, Munksgaard L, Humphries D, Kennedy E, Dijkstra J, Dewhurst R, Ferguson H, Terré M, Kuhla B, (editors). Methods in cattle physiology and behaviour – Recommendations from the SmartCow consortium. Cologne: PUBLISSO; 2020. DOI: 10.5680/mcpb007 (//books.publisso.de/en/publisso_gold/publishing/books/overview/53/188).

The results of the joint research activities for WP5 highlight key aspects of the procedures described and specific sources of variation that warrant further investigation, but do not suggest that major revisions of the current chapter are required. Some of the key considerations for reducing variance and increasing the minimum detectable difference include:

General:

1. Strict attention to detail and protocols is critical to minimizing variation in the measurements obtained. Improvements can be achieved through routine self assessment and critical evaluation at each installation. These evaluations should be conducted at each experimental facility as the application of specific methods and procedures will vary due to the historical use of procedures and modifications that have occurred over time.
2. Training of staff and students conducting trials and effective communication is essential to be sure all are fully engaged and committed to the success of the experiments and are aware of the implications of specific procedures and sources of variation.

Specific procedures:

1. Under conditions of measurement at INRAE repeatability of measurements of digestion and N balance of growing beef cattle were greater after at least 7 days of collection and for digestibility was greatest after 10 days of collection.
2. Shorter periods of 4 days were acceptable in a trial with lactating dairy cows at Reading where cows were adapted to digestion stalls before experiments began and intake and diet composition did not vary over the course of measurement periods. The current EU directive is for collection periods to not exceed 5 days although a derogation can be obtained if justified.
3. Cow comfort and adaptation and training to digestion stalls and collection equipment and procedures is important for reducing variability and minimizing stress.
4. Accurate measurement of diet intake and composition of feeds and refusals is critical.
5. It is essential that collection of faeces and urine is complete and no cross contamination occurs. Mixing of acid and urine throughout collection is highly recommended.
6. Frozen bulk samples of feed and faeces are acceptable for studies of N digestion and excretion if processed in a manner that minimizes volatile N losses. In this regard analysis of samples that have been subjected to minimal processing and kept chilled or frozen during chopping and mixing is essential. This can be achieved by mixing samples with dry-ice or liquid N. Samples should not be dried before analysis unless volatile N losses can be accounted for.

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7. Addition of acid to faeces or manure in these studies was not effective as a preservative and reduced N concentration of faeces and manure.
8. Urine should be analyzed fresh if possible, otherwise the time spent frozen before analysis should be kept as short as possible.

Factors needing further investigation:

1. Reasons for the apparent loss of N from frozen urine samples need to be determined and alternative approaches identified.
2. Reasons for the higher feed N concentration obtained using Dumas combustion compared to Kjeldahl at WU need to be identified and addressed.
3. Losses of ammonia during collection should be quantified if possible, especially under conditions where there is mixing of urine and faeces during collection In this regard procedures for ammonia emission measuring should be standardized and recovery tests conducted.

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8) Appendix

8.1) Appendix 1: MS5.1 Evaluation of historical data for variation in digestion and N balance data.

SmartCow Summary of Results – N Balance Meta-analysis

Introduction

Measurements of feed efficiency related to a 'sustainable phenotype' for cattle include feed digestion, nitrogen (N) excretion in faeces, urine, and milk, and N balance. However, it is important to 'refine' the techniques used for these measurements in order to minimize technique associated variation and thereby 'reduce' the number of animals required for our experiments when alternative approaches such as proxies and biomarkers (WP6) are not appropriate (Hristov et al., 2019). Moreover, from an environmental perspective, accurate estimation of N retention is of large importance because dietary N consumed and not retained in animal tissues or secreted in milk is excreted in urine and faeces. This contributes to water pollution, gaseous N emissions including ammonia and nitrous oxide, and small particulate matter formation in the atmosphere (Bougouin et al., 2022). Urinary N is much more labile and susceptible to fast leaching and volatilization losses than faecal N, and therefore accurate estimation of faecal vs urine N excretion is required. Thus key measurements are whole digestive tract diet digestion and associated measurements of N excretion in faeces and urine required (with milk N excretion measurement if needed) to measure whole body N utilization, typically based on total collection of urine and faeces, with separation of faeces and urine maintained to minimize volatile N losses during collection (Schneider and Flatt, 1975; Hristov et al., 2019). Whilst these measurements have a long history of use, there is wide variation in the standard operating procedures in use at individual locations and it has long been known that measurements of N balance are often subject to large errors of measurement (Hristov et al., 2019; Martin, 1966; Sphanghero and Kowalski, 1997 and 2021). Known sources of measurement error include cross-contamination of faeces and urine, which can lead to urea catabolism and ammonia loss, N losses during sample preparation, storage, drying, and grinding prior to analysis, insufficient mixing of urine and acid added during collection to minimize ammonia loss, and the number of days of collection. Historically collection periods of 10 days or more were used for digestion trials in dairy cattle to account for daily variations in faecal output, whilst more recently collection period of 4 days have become common, as this negates the need for weekend work.

Therefore, our objective was to assemble a data base of N balance measurements for cattle and associated diet and production variables and conduct a meta-analysis to determine the extent to which variation in measurements is attributable to research location and the methods used, after accounting for variation due to feed DMI and composition and other animal characteristics that may affect N excretion and tissue balance. This will provide an evidence base for recommendations of 'best practice' at SmartCow research facilities that increase precision and accuracy and minimize animal numbers required for 'in vivo' measurements of N balance.

Methods

Database

The complete data set was comprised of 3969 individual cow measurements of diet digestion and N excretion and balance from digestion trials carried out at 15 research sites associated with 11 different research institutions. The data base was assembled using data from SmartCow (https://www.smartcow.eu/) partners as well as participants in the Food and Nutrition Network of the Global Alliance

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(https://globalresearchalliance.org/research/livestock/networks/feed-nutrition-network/). Research sites were located in North America (3), Europe (11) and Oceania (1). The data set was comprised of 125 separate experiments with sites contributing data from 1 to 34 experiments (18 to 759 observations). Individual cow measurements included dry matter intake (DMI), chemical composition of the diets fed, nutrient intakes, faecal DM and N outputs, urinary N excretion, milk yield and composition, and body weight and other animal characteristics (e.g. breed, physiological state, sex). Retained N balance was calculated by subtracting faecal N, urine N and milk N (if lactating animals) excretions from N intake. Complete N balance data was available for 12 sites allowing the calculation of N balance for these observations (2835 observations). Figure 1 summarises the entire data set and subsequent subsets of data used in the data analysis processes.

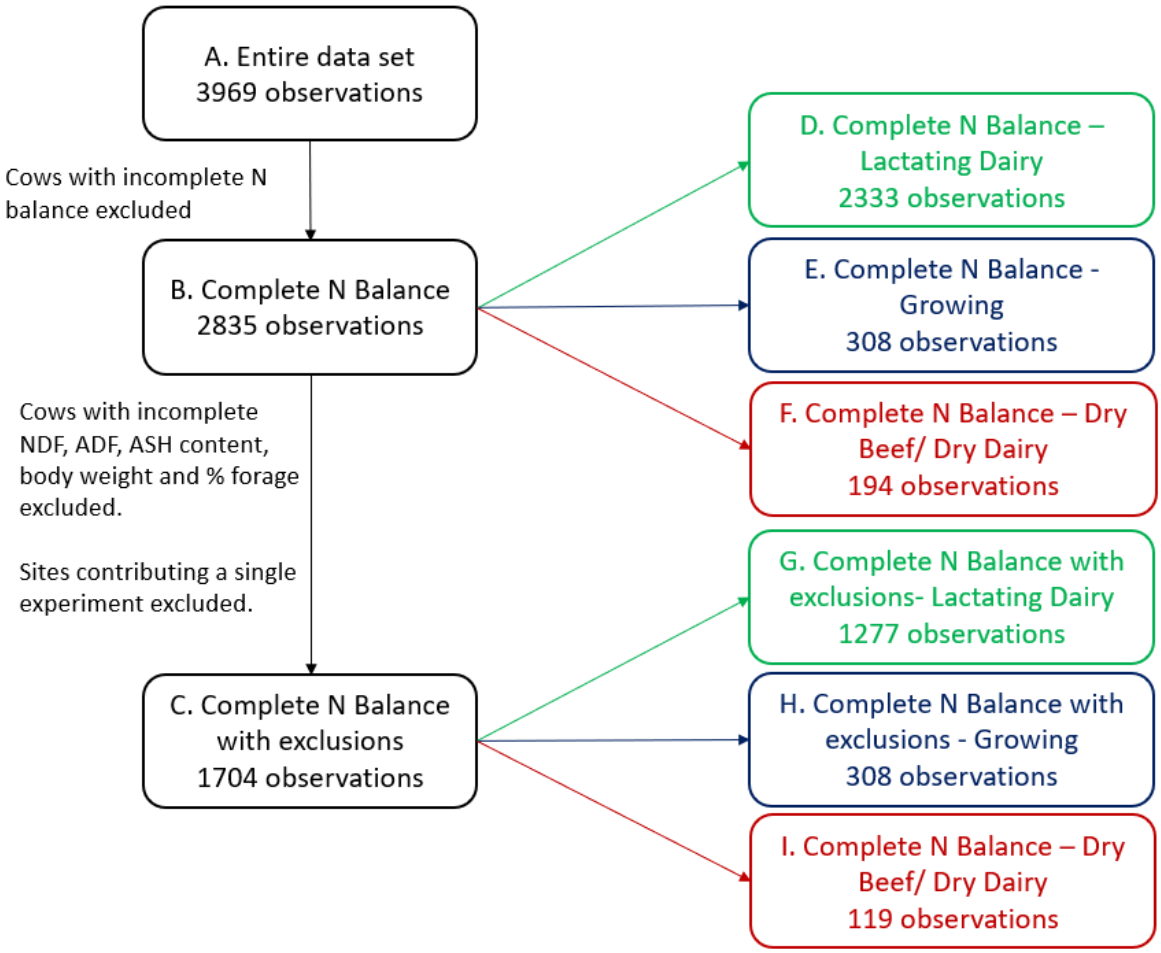

Figure 1 – Summary data sets considered in the modelling processes with details of data exclusions made.

Analysis of single explanatory variables

Variables with a high proportion of missing observations (>60% of the total number of individual cow measurements) were excluded from models used in further analyses. It was considered important that dietary N intake was accounted for in the models as the key determinant of N excretion in faeces, urine and milk. To achieve this, each response variable (retained N, urine N excretion, faecal N excretion, milk N) was tested with either: 1) N intake or 2) DMI and CP content as explanatory variables. The model fit for the N intake models was better based on AIC and BIC values, so NI was included in all subsequent models of single explanatory variables. First, a correlation matrix was produced for numerical variables to identify highly correlated variables, $|r| = 0.75$ (Appendix 1). DMI and N intake were highly correlated to a number of other dietary intake measures, as DMI determined the total intake of all diet components. Therefore, the decision was made to take forward N intake and diet ash, NDF, ADF and starch concentrations for further analysis along with a range of methodology-related variables.

Methodological variable categories were: treatment type (diet change, feed additive, abomasal infusion, delivery method, cow status), experimental design (change over, randomised), DMI measurement method (weighed manually or electronically), feed presentation (total mixed ration, separate forage and concentrate, pellets, cut and carry), and collection method (total collection, manure – faecal N, faecal marker and spot collection of urine, faecal marker with no urine collection), This reduced selection of relevant diet composition, animal characteristic, and methodological variables (Table 1) were tested individually in a series of mixed models where retained N, urine N excretion, faecal N excretion or milk N excretion were the outcome variables. Experiment nested within research site were random effects unless there was insufficient variation to support nesting experiment within site. Variables were selected for further model development if their effect was significant or had a nonsignificant trend ($P < 0.10$). Table 1 summarises the results of the single variable (plus N intake) analysis and shows the short list of variables considered for further analysis. Methodological variables (Table 2) included treatment type (e.g. diet change or feed additive), experimental design (continuous or change over), duration of adaptation to treatment, days of collection, N analysis method (Kjeldahl or combustion), DMI measurement method (manual or automated weighing), intake level (ad lib vs restricted), feed presentation (e.g. total mixed ration [TMR], pellets, etc.), and collection method (e.g. total or marker).

Table 1. Summary of single variable analysis where ✓= p<0.05, p=0.1-0.05 = NST and × = p>=0.1

	Retained N	Urine N excretion	Faecal N excretion [1]	Milk N excretion (Dairy only subset)
N intake	✓	✓	✓	✓
Cow status	✓	✓	✓	NA
Treatment type	×	✓	×	×
Experimental design	×	✓	✓	×
Adaptation duration	×	×	×	×
Duration of collection	×	× [1]	×	NST
N analysis method	NST	×	NST	✓ [1]
DMI Measurement method	NST	✓	×	✓
Feed restriction	✓	✓	×	×
Feed presentation	×	✓	×	✓
Collection method	×	×	×	×
Lactation	✓	✓	✓	✓
Sex	×	×	×	NA
Body weight	✓	✓	×	✓
Ash content	✓	✓	✓	✓
NDF content	×	×	✓	✓
ADF content	×	×	✓	✓
Diet forage proportion	×	×	×	✓

[1] random effect of experiment used as insufficient variation to support the nesting of experiment within site

Multivariate Analysis

Outcome variables were retained N (N balance), urine N excretion, faecal N excretion or milk N excretion. Each were tested in a linear mixed models with experiment nested within site as random effects with random intercepts. An automated model build function based on lowest AIC while controlling for variance inflation factors was used to determine the 'best model' fit using R version 4.0.3 (R Core Team, 2020; van Lingen et al., 2019). Variables from table 1 that were p<0.10 for one of more of the outcome variables were taken forward for the model build process. Highly correlated variables identified in the correlation matrix i.e. ADF content and NDF content were prevented

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from appearing in the model at the same time as each other. The inclusion of experiment duration and N chemistry method (i.e. Dumas or Kjeldahl) in combination with other variables resulted in high VIF. As experiment duration was non-significant for retained N, urine N and faecal N and only resulted in a non-significant trend for milk N it was decided to include N chemistry method in the short list of variables for the model build in which all combinations of the following variables were tested: N intake, cow status, body weight, diet ASH content, diet NDF content, diet ADF content, experiment design, DMI method, N chemistry method, feed offered (e.g. ad lib or restricted), collection method, treatment type, feed presentation, and forage proportion. Parity was only included in the short list for the milk N models. The sum of the variance associated with each random effect (site and experiment) and the residual variance were used to calculate a percentage variance associated with each.

Results

Descriptive summaries

The complete data set includes lactating beef cattle, growing cattle and dry dairy cattle but experiments with lactating dairy cattle dominate the data set and as a result the majority (85%) of animals are multiparous females (table 2). After lactating dairy, growing cattle was the next largest animal status category accounting for 10% of the total data set. However, many of the animal and methodology variables contained categories with no or very few data points reported, precluding the use of the growing animal only data set to determine causes of variation in the measurement of N balance. Beef breeds were not requested in the original data gathering exercise, so it is not possible to determine differences between other non-specified dairy breeds and beef breeds. The methodologies used in the experiments are summarised in table 2. Latin square and randomised experimental designs were dominant as was total collection of urine and faeces.

Table 2. Summary of categorical data

Categories	Entire data set n (%)	Complete N balance n (%)	Complete N Balance – Lactating Dairy n (%)	Complete N Balance – Growing n (%)
Animals				
Cow Status				
Dry dairy	160 (4.0)	160 (5.6)	0 (0.0)	0 (0.0)
Growing	408 (10.3)	308 (10.9)	0 (0.0)	308 (100.0)
Lactating beef	34 (0.9)	34 (1.2)	0 (0.0)	0 (0.0)
Lactating dairy	3367 (84.8)	2333 (82.3)	2333 (100.0)	0 (0.0)
Breed				
Holstein Friesian	2897 (73.0)	2019 (71.2)	1795 (76.9)	91 (29.5)
Jersey	58 (1.5)	58 (2.0)	42 (1.8)	0 (0.0)
Ayrshire	112 (2.8)	88 (3.1)	88 (3.8)	0 (0.0)
Brown Swiss	245 (6.2)	132 (4.7)	120 (5.1)	12 (3.9)
HF x Jersey	12 (0.3)	12 (0.4)	12 (0.5)	0 (0.0)
Other	373 (9.4)	277 (9.8)	27 (1.2)	205 (66.6)
Unknown	272 (6.9)	249 (8.8)	249 (10.7)	0 (0.0)
Sex				
Male	187 (4.7)	91 (3.2)	0 (0.0)	91 (29.5)
Female	3587 (90.4)	2549 (89.9)	2333 (100.0)	56 (18.2)
Unknown	195 (4.9)	195 (6.9)	0 (0.0)	161 (52.3)
Lactation category				
Multiparous	2185 (55.1)	1582 (55.8)	1482 (63.5)	0 (0.0)
Not-applicable	468 (11.8)	368 (13.0)	0 (0.0)	308 (100.0)
Primiparous	552 (13.9)	302 (10.7)	290 (12.4)	0 (0.0)

Unknown	764 (19.2)	583 (20.6)	561 (24.0)	0 (0.0)
Methodologies				
Type of experiment				
Change over	2745 (69.28)	1809 (63.8)	1481 (63.5)	280 (90.9)
Randomised trial	1204 (30.3)	1006 (35.5)	832 (35.7)	28 (9.1)
Unknown	20 (0.5)	20 (0.7)	20 (0.9)	0 (0.0)
Duration of adaptation				
0-6 days	61 (1.5)	60 (2.1)	60 (2.6)	0 (0.0)
7-15 days	1117 (28.1)	832 (29.3)	801 (34.3)	12 (3.9)
Over 15 days	1352 (34.1)	596 (21.0)	564 (24.2)	0 (0.0)
unknown	1436 (36.3)	1347 (47.5)	908 (38.9)	296 (96.1)
Duration of collection				
2 days	108 (2.7)	72 (2.5)	72 (3.1)	0 (0.0)
3 days	93 (2.3)	92 (3.2)	92 (3.9)	0 (0.0)
4 days	782 (19.7)	137 (4.8)	137 (5.9)	0 (0.0)
5 days	928 (23.4)	856 (30.2)	729 (31.2)	44 (14.3)
6 days	564 (14.2)	506 (17.8)	495 (21.2)	0 (0.0)
7 days	976 (24.6)	866 (30.5)	522 (22.4)	252 (81.8)
8 days	370 (9.3)	158 (5.6)	138 (5.9)	12 (3.9)
Unknown	148 (3.7)	148 (5.2)	148 (6.3)	0 (0.0)
Intake method				
Weighed electronically	900 (22.7)	140 (4.9)	140 (6.0)	0 (0.0)
Weighed manually	3069 (77.3)	2695 (95.1)	2193 (94.0)	308 (100.0)
Wet chemistry methods				
Dumas All	1620 (40.8)	746 (26.3)	710 (30.4)	12 (3.9)
Kjeldahl All	1867 (47.0)	1663 (58.7)	1229 (52.7)	296 (96.1)
Mixture	270 (6.8)	266 (9.4)	234 (10.0)	0 (0.0)
NIR	12 (0.3)	12 (0.4)	12 (0.5)	0 (0.0)
unknown	200 (5.0)	148 (5.2)	148 (6.3)	0 (0.0)
Collection method				
Faecal marker no urine	602 (15.2)	0 (0.0)	0 (0.0)	0 (0.0)
Faecal marker urine spot	162 (4.1)	72 (2.5)	72 (3.1)	0 (0.0)
Manure minus Faecal marker	214 (5.4)	158 (5.6)	158 (6.8)	0 (0.0)
Total collection	2991 (75.4)	2605 (91.9)	2103 (90.1)	308 (100.0)

A summary of diet composition, nutrient intake, diet digestibility and nitrogen outputs and retention variables are presented in table 3. Summaries of the additional data subsets were also produced but are not presented here as there were few discernible differences in mean values. The data were reviewed and checked for data points outside of expected ranges. Where potential errors were identified or where values were not considered biologically plausible, contributors were contacted to check their data sets and confirm any known reasons why the data should be excluded. As this aim of this meta-analysis was to consider sources of variation in the measurement of nitrogen excretion in cattle all data were retained unless there was a known error.

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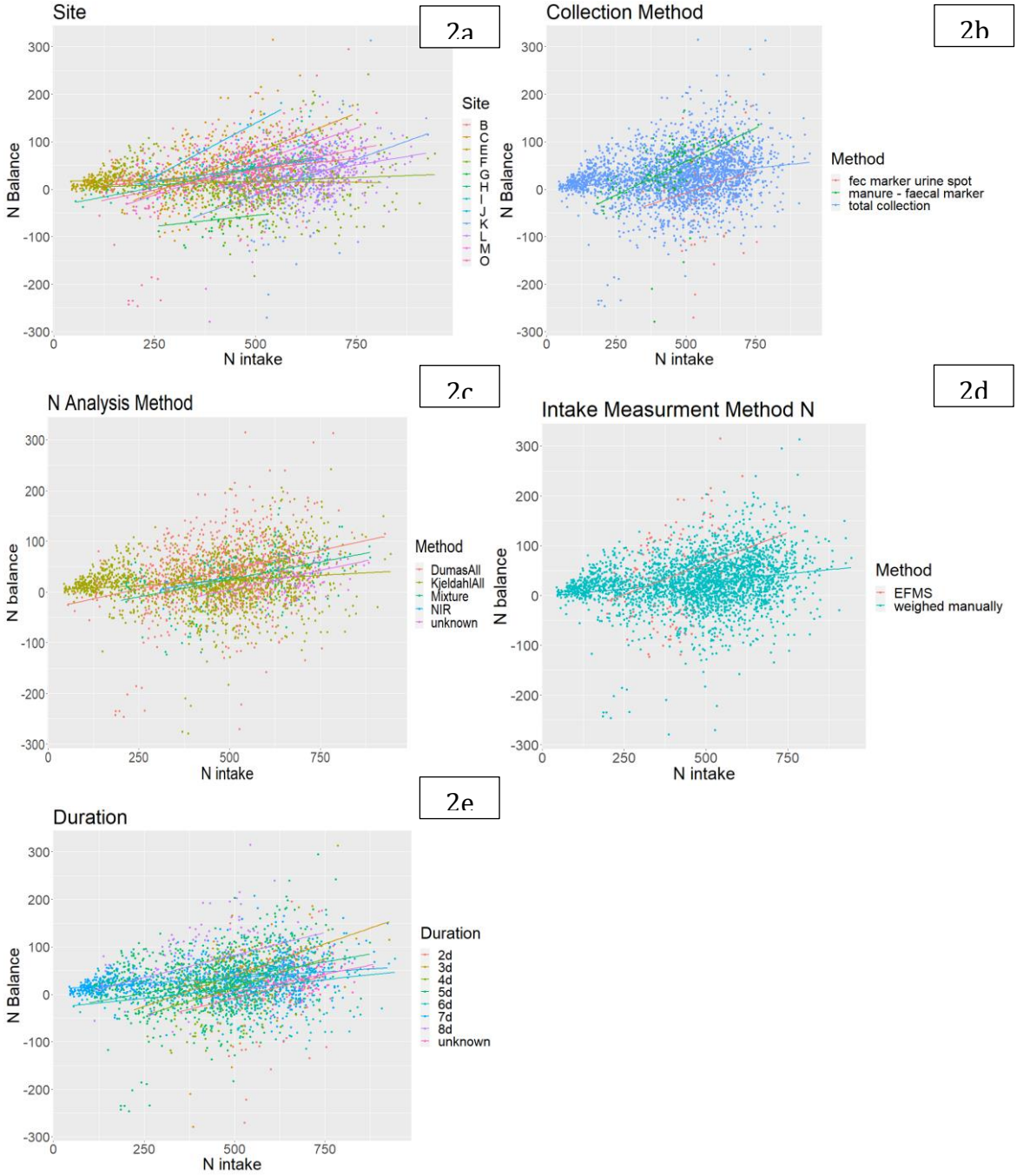
Table 3. Summary of diet composition, nutrient intake, diet digestibility and nitrogen outputs and retention for the entire study data set (3969 observations).

	Mean	SD	Min	Max	CV	n
Diet Composition						
CP concentration (g/kg DM)	163	26	71	435	0.16	3932
EE concentration (g/kg DM)	35	13	7	88	0.37	1919
ASH concentration (g/kg DM)	75	17	28	154	0.22	3367
NDF concentration (g/kg DM)	350	69	136	655	0.20	3455
ADF concentration (g/kg DM)	209	51	66	397	0.24	2575
Starch concentration (g/kg DM)	232	116	1	641	0.50	2471
Nutrient intakes						
DM intake (kg/d)	18.4	6.2	2.1	37.5	0.33	3962
Forage proportion (% diet DM)	60.1	16.4	4.7	100.0	0.27	3360
CP intake (kg/d)	3.1	1.1	0.3	7.2	0.37	3377
N intake (g/d)	493	180	43	11528	0.36	3735
EE intake (kg/d)	0.62	0.39	0.02	2.19	0.62	1692
ASH intake (kg/d)	1.4	0.5	0.1	4.6	0.38	3013
NDF intake (kg/d)	6.6	2.1	0.4	13.9	0.32	3165
ADF intake (kg/d)	3.8	1.5	0.2	9.5	0.39	2532
Starch intake (kg/d)	4.1	1.8	0.0	12.3	0.45	2257
Diet digestibility						
DM digestibility (%)	69.7	6.8	22.9	87.0	0.10	3142
OM digestibility (%)	71.8	4.5	52.4	88.4	0.06	2628
N digestibility (%)	66.7	6.8	27.4	88.5	0.10	3026
NDF digestibility (%)	55.5	10.9	4.4	91.2	0.20	2908
ADF digestibility (%)	52.2	12.3	5.0	92.6	0.24	2037
Starch digestibility (%)	95.8	4.6	43.4	100.0	0.05	1791
Nitrogen output and retention *(using observations with complete N balance data sets only)*						
Total N excretion (g/d)*	500	130	167	936	0.26	2333
Faecal N excretion (g/d)	156	65	11	385	0.41	2835
Urine N excretion (g/d)	159	73	4	446	0.46	2835
Milk N (g/d)*	151	42	41	296	0.28	2333
Retained N (g/d)	28	53	-279	315	1.94	2835
Faecal N as % of N intake	34.8	7.6	10.6	93.5	0.22	2835
Urine N as % of N intake	36.0	13.6	2.2	99.3	0.38	2835
Manure N as % of N intake	69.7	13.9	28.0	167.8	0.20	2835
Milk N as % of N intake*	29.2	6.8	9.3	65.7	0.23	2333
Retained N as % of N intake	6.1	13.4	-130.6	57.9	2.18	2835
Animal Data						
Body weight (kg)	612	126.3	129.0	974.0	0.21	3279
Milk production (kg/d)*	29.9	9.1	5.9	63.5	0.30	2310
Days in Milk (at exp start)*	137	78.7	4	567	0.61	1448

*Lactating dairy only

Variation associated with site and key methodologies are visualised in figures 2a-e (N balance), 3a-e (urine N) and 4a-e (Faecal N).



Figures 2a-e. Data visualisations of N intake v N Balance plotted with regression lines for location and methodology classifications.

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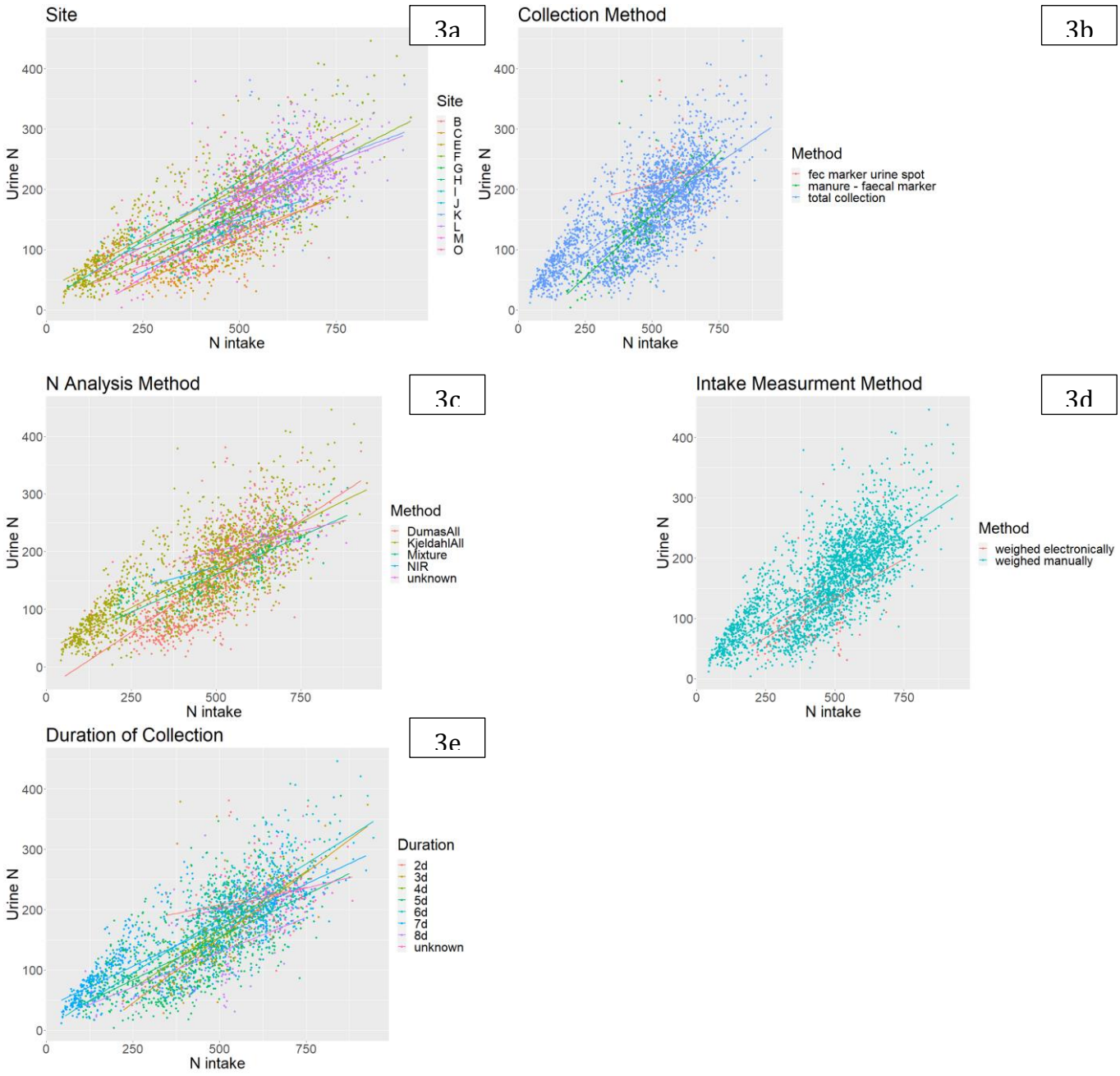
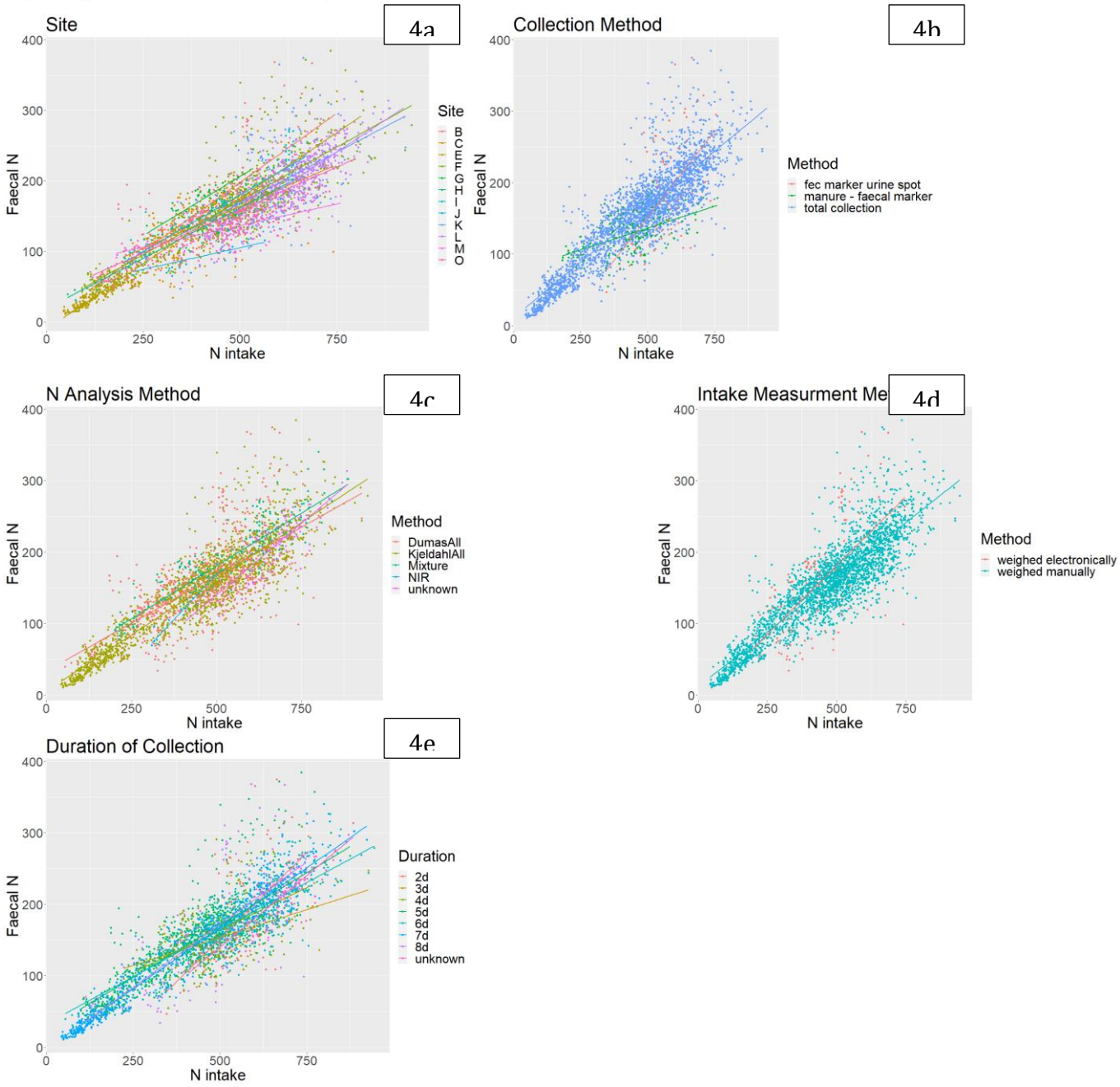


Figures 3a-e. Data visualisations of N intake vs. Urine N plotted with regression lines for location and methodology classifications.



Figures 4a-e. Data visualisations of N intake vs. Faecal N plotted with regression lines for location and methodology classifications.

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Retained N models

The best models for retained N for the maximal all animal data set (B), the all animal data set with exclusions (C) and the lactating cow only data set with exclusions (G) are presented in tables 4a-c respectively (see Figure 1). While the models presented in tables 4a and 4b contain the same variables the significance and effect sizes vary for the status variable. Where the model is produced for the lactating dairy cow only data set body weight and diet ash content are retained in the best model. Site level variation ranges from 21-27% across all models.

Table 4a. Best linear mixed effects model for retained N using data set B, maximum observations.

		Coefficient	SE	df	T-value	P value
Intercept		-18	10	15	-1.770	0.097
N intake		0.24	0.01	2813	26.925	<0.001
Status	Ref: dry dairy					
	Growing	25.24	15.85	119	1.592	0.114
	Lactating beef	-2.41	18.87	228	-0.127	0.899
	Lactating dairy	-82.04	4.88	2808	-16.814	<0.001

No. observations = 2817, No. of experiments = 106, No. of Sites = 11,
random effect = site|experiment,
Model variance: experiment = 37.9%, site = 21.1%, residual 40.9%

Table 4b. Best linear mixed effects model for retained N using data set C, with exclusions.

		Coefficient	SE	df	T-value	P value
Intercept		-29	12	19	-2.47	0.024
N intake		0.21	0.01	1698	19.32	<0.001
Status	Ref: dry dairy					
	Growing	49.88	13.91	86	3.59	<0.001
	Lactating beef	24.80	16.88	172	1.47	0.144
	Lactating dairy	-46.95	7.45	1475	-6.31	<0.001

No. observations = 1704, No. of experiments = 61, No. of Sites = 9,
random effect = site|experiment,
Model variance: experiment = 27.8%, site = 26.6%, residual 45.6%

Table 4c. Best linear mixed effects model for retained N using data set G, lactating dairy cows only with exclusions.

	Coefficient	SE	df	T-value	P value
Intercept	88	18	98	-4.856	<0.001
N intake	0.226	0.014	1263	16.370	<0.001
Body weight	-0.059	0.018	1275	-3.345	<0.001
Ash content	0.609	0.141	1167	4.323	<0.001

No. observations = 1277, No. of experiments = 49, No. of Sites = 9,
random effect = site|experiment
Model variance: experiment = 28.2%, site = 22.9%, residual 48.8%

Urine N models

The best models for urine N for the maximal all animal data set (B), the all animal data set with exclusions (C), and the lactating cow only data set with exclusions (G) are presented in tables 5a-c respectively (Figure 1). All models

contain the same variables, and the results are highly consistent between models. Site level variation ranges from 20-25% across all models.

Table 5a. Best linear mixed effects model for urine N excretion using data set B, maximum observations.

	Coefficient	SE	df	T-value	P value
Intercept	-22	10	22	-2.10	0.048
N intake	0.26	0.01	2256	34.94	<0.001
Body weight	0.10	0.01	2272	9.73	<0.001

No. observations = 2274, No. of experiments = 82, No. of Sites = 11,
random effect = site|experiment,
Model variance: experiment = 42.7%, site = 20.3%, residual 37.0%

Table 5b. Best linear mixed effects model for urine N excretion using data set C, with exclusions

	Coefficient	SE	df	T-value	P value
Intercept	-26	11	20	-2	0.0313
N intake	0.26	0.01	1638	29	<0.001
Body weight	0.11	0.01	1703	10	<0.001

No. observations = 1704, No. of experiments = 61, No. of Sites = 9,
random effect = site|experiment,
Model variance: experiment = 35.5%, site = 23.1%, residual 41.4%

Table 5c. Best linear mixed effects model for urine N excretion using data set G, lactating dairy cows only with exclusions.

	Coefficient	SE	df	T-value	P value
Intercept	-35	14	28	-2.50	0.019
N intake	0.28	0.01	1276	22.84	<0.001
Body weight	0.10	0.02	1270	6.26	<0.001

No. observations = 1277, No. of experiments = 49, No. of Sites = 9,
random effect = site|experiment,
Model variance: experiment = 33.1%, site = 24.8%, residual 42.1%

Faecal N models

A lack of variation prevents the use of experiment nested within site with faecal N as the response variable. Therefore, the best models for faecal N for the maximal all animal data set (B), the all-animal data set with exclusions (C) and the lactating cow only data set with exclusions (G) are presented for a models with experiment as a random effect in tables 6a-c.

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Table 6a. Best linear mixed effects model for faecal N excretion using data set B, maximum observations

		Coefficient	SE	df	T-value	P value
Intercept		16	4	351	4.39	<0.001
N intake		0.28	0.00	2817	56.69	<0.001
Status	Ref: dry dairy					
	Growing	-7.17	9.23	119	-0.78	0.439
	Lactating beef	-4.74	10.70	209	-0.44	0.658
	Lactating dairy	15.66	2.65	2813	5.90	<0.001

No. observations = 2817, No. of experiments = 106, random effect = experiment
Model variance: experiment = 62.4%, residual 37.6%

Table 6b. Best linear mixed effects model for Faecal N excretion – data set C, with exclusions

		Coefficient	SE	df	T-value	P value
Intercept		0	5	253	-0.03	0.974
N intake		0.27	0.01	1697	42.96	<0.001
Status	Ref: dry dairy					
	Growing	8.93	9.32	79	0.96	0.341
	Lactating beef	11.35	10.78	138	1.05	0.294
	Lactating dairy	30.17	4.39	1648	6.88	<0.001

No. observations = 1704, No. of experiments= 61, random effect = experiment
Model variance: experiment = 58.7%, residual 41.3%

Table 6c. Best linear mixed effects model for Faecal N excretion – data set G, lactating dairy cows only with exclusions

	Coefficient	SE	df	T-value	P value
Intercept	32	10	744	3.20	<0.001
N intake	0.28	0.01	1265	36.88	<0.001
Ash content	-0.38	0.08	1252	-4.51	<0.001
ADF content	0.11	0.03	1198	3.40	<0.001

No. observations = 1277, No. of experiments = 49, random effect = experiment
Model variance: experiment = 56.0%, residual 44.0%

Milk N models

The lactating cow only data set with exclusions (G) was used to produce a best model for milk N and is presented in table 7d. Parity was also retained in the best model for milk N with primiparous cows having lower milk N than multiparous cows. Site accounted for 34% of the variation.

Table 7d. Best linear mixed effects model for Milk N – data set G, lactating dairy cows only with exclusions

		Coefficient	SE	df	T-value	P value
Intercept		121	12	74	10.44	<0.001
N intake		0.22	0.01	1271	30.67	<0.001
Body weight		-0.05	0.01	1268	-5.22	<0.001
Ash content		-0.50	0.07	1258	-6.79	<0.001
ADF content		-0.11	0.03	1149	-4.12	<0.001
Parity	Ref: Multiparous					
	Primiparous	-12.17	2.50	1164	-4.87	<0.001
	Unknown	9.77	5.53	533	1.77	0.078

No. observations = 1277, No. of experiments = 49, No. of Sites = 9,
random effect = site|experiment,
Model variance: experiment = 26.3%, site = 34.3%, residual 39.4%

Discussion

As observed in previous analyses (e.g. Angelidis et al., 2019; Bougouin et al., 2022), N intake was the major determinant of N excretion in faeces, urine and milk of cattle in the present study. When data from growing and nonlactating cattle are included in the data analysed, cattle type also had an effect on N excretion in faeces and N retention, with greater relative amounts of faecal N excretion and less body N retention for lactating dairy cows compared to growing, dry and lactating beef cattle. For faecal N excretion this may reflect effects of higher levels of DMI and diets with higher digestibility that result in greater faecal outputs, including greater endogenous faecal N associated with increased hindgut fermentation of carbohydrates in lactating dairy cows. For urine N excretion, body weight was the only significant factor beyond N intake in the present analysis, with a positive association between body weight and urine N excretion, which could reflect effects of total body protein turnover and 'non-productive' protein requirements. Yan et al. (2006) reported that body weight improved prediction of manure N excretion slightly when included with N intake in prediction models, but was a relatively poor predictor or manure N excretion on its own or in combination with milk yield. For lactating dairy cows only, both diet ash and ADF concentration were significantly associated with faecal N excretion. Diet ADF had a positive effect, perhaps also reflecting increased hindgut fibre fermentation or excretion of indigestible protein fractions. Diet ash concentration had a negative association with faecal and milk N excretion in lactating dairy cows, and also had a positive association with retained N. This suggests that diets with higher ash levels were associated with lower milk protein yield and thus relatively more body N retention, but the mechanisms involved are impossible to ascertain based on the diet composition data available in the current study. There was also a negative association between body weight and milk N excretion, as well as N retention in the dairy cow data base. Reasons for these associations between body weight and N excretion and retention, whereby larger cows excrete less dietary N increments in milk and retain less increased N intake in body tissues are not certain, but in a previous meta-analysis of N excretion and retention in growing beef cattle there was also a negative association between body weight and retained N as a proportion of N intake (Agelidis et al., 2019).

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Initial visualisations of the data (figs 2a-e, 3a-e, 4a-e) suggested there to be variation associated with site and a number of methodologies associated with the measurement of nitrogen balance, however none of the methods identified were significant in the mixed models analysis and thus were not retained in the series of 'best' models produced for retained N, urine N or faecal N. A number of explanations exist for this apparent lack of relationship between methods and N excretion. For a number of the variables there was a dominant method in use and relatively few observations in the remaining category or categories such as, the measurement of DMI where manual weighing accounted for more than 95% of observations and total collection of urine and faeces accounted for 92% of observations (table 1). In addition, these more uncommon methodologies were often only undertaken at a limited number of locations leading to confounding with site e.g. electronic feeding was only used in trials at 2 locations. Confounding with site was likely to have been a significant problem with the variable duration of collection, where many locations favour a single duration of collections and there is a lack of within site variation. The number of observations for 2, 3, 4 and 8 days collections are very small (table 1) so it is not possible to infer from this database if the variation observed at the extremes of collection periods is associated with variation in N measurement (figs 2e, 3e, 4e). There is relatively less variation associated with the more common collection durations of 5-7 days.

For all models over half of the variation was associated with site and experiment. Experiment variation ranged from 28-37%, for retained N, 33-42% for urine N and was 26% for Milk N. Site variation ranged from 21-27%, for retained N, 20-25% for urine N and was 34% for Milk N. Site level variation was less for faecal N measurement (fig 4a) and this variation was interrelated with experiment variation, so it was not possible to run the models with experiment nested within experiment as a random effect. Experiment level variation was 56-62% for faecal N but the residual model variation was similar to that for the models of retained, urine and milk N. When compared with the site level variation for observed for methane measurement of 7% obtained from a similar database meta-analysis, the site level variation associated with the measurement of N excretion and balance is greater, reflecting the complexity of the data collection with multiple measurements begin taken, some manually, in order to calculate the final values of N intake, urine N faecal, urine and milk N excretion, and finally retained N. For milk N excretion, the variation across sites likely reflects differences in milk yield of the cows used in the studies included.

For the methodology related variables, it is highly likely that there is a finer level of detail that was not captured adequately e.g. the N analysis method variable did have detail on how the faecal and urine samples were gathered, stored and prepared for analysis, and it is known that drying and grinding can result in volatile N loss from samples (Sphanghero and Kowalski, 1997; Morris et al., 2019). Also not captured was any data regarding the influence of human behaviour with regards to the methods used, which would be a significant challenge to collect but has the potential to have a considerable influence on N balance measurements. The ability to identify individual sources of variation that explain the high site level variation in N balance measurements was likely affected by lack of within site variation in methodologies, insufficient data for some methodologies, insufficient detail for some methodologies, and human behaviour in the conduct of the methods used. It is likely that a multi-site experimental protocol including a range of defined methodologies would be needed to successfully unpick the interactions between site and methodology.

Conclusions

As observed previously, dietary N intake is the major determinant of N excretion in faeces, urine and milk, but other dietary and animal factors modify the effects of N intake to varying degrees, ultimately reflecting effects of diet component intakes and nutrient supply relative to requirements. The elucidation of these factors was not the objective of the present meta-analysis, but the effects of diet and animal factors were accounted for in order to evaluate variation in measurements of N balance associated with methodology and research site. Considerable variation in measurements of N excretion and retention due to research site was observed, but this variation across sites was not due to the specific methodologies assessed. The present analysis suggests that in addition to differences in the production of the experimental animals studied, variation across sites may be due more to how the specific experimental methods are used, rather than the method used per se. In this regard strict attention to detail in the conduct of methods and procedures and training of staff and students undertaking the experimental

procedures at all levels, from feeding and collection of samples, through to sample storage, processing and analysis is critical (Snedecor and Flatt, 1970).

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Appendix 1. Correlation matrix for numerical variables (R^2 >0.5 = yellow, R^2 >0.75 = red)

	DMI	*Forage %*	*CP cont*	*EE cont*	*ASH cont*	*NDF cont*	*ADF cont*	*STA cont*	*CP intake*	*N intake*	*EE intake*	*ASH intake*	*NDF intake*	*ADF intake*	*STA intake*	*DM dig*	*OM dig*
DMI	1.00																
pcFor	0.00	1.00															
CP cont	0.22	-0.07	1.00														
EE cont	0.23	0.02	0.14	1.00													
ASH cont	0.00	0.29	0.23	-0.01	1.00												
NDF cont	-0.12	0.43	-0.17	-0.03	0.46	1.00											
ADF cont	0.07	0.48	-0.08	0.04	0.44	0.84	1.00										
STA cont	-0.41	-0.59	-0.21	-0.23	-0.43	-0.51	-0.65	1.00									
CP intake	0.93	0.01	0.54	0.25	0.07	-0.21	0.00	-0.43	1.00								
N intake	0.92	0.01	0.35	0.25	0.08	-0.20	0.00	-0.43	1.00	1.00							
EE intake	0.74	0.11	0.18	0.83	-0.03	-0.02	0.13	-0.42	0.72	0.72	1.00						
ASH intake	0.82	0.14	0.35	0.24	0.39	0.01	0.22	-0.50	0.84	0.84	0.68	1.00					
NDF intake	0.85	0.22	0.11	0.20	0.13	0.32	0.43	-0.56	0.76	0.76	0.64	0.76	1.00				
ADF intake	0.84	0.25	0.12	0.24	0.15	0.29	0.52	-0.62	0.75	0.75	0.67	0.76	0.93	1.00			
STA intake	0.65	-0.14	0.04	-0.01	-0.23	-0.36	-0.25	0.23	0.60	0.60	0.32	0.51	0.44	0.42	1.00		
DMdig	-0.10	-0.20	0.11	-0.01	-0.13	-0.38	-0.47	0.24	-0.03	-0.03	-0.08	-0.12	-0.27	-0.32	-0.16	1.00	
OMdig	-0.04	0.14	0.24	0.13	0.09	-0.12	-0.33	-0.15	0.08	0.08	0.00	0.01	-0.15	-0.30	-0.12	0.66	1.00
Ndig	0.02	-0.01	0.47	0.08	0.11	-0.11	-0.10	0.03	0.17	0.17	0.07	0.03	-0.08	-0.06	-0.04	0.37	0.53
NDFdig	-0.05	0.24	0.22	0.16	0.38	0.33	0.07	-0.31	-0.03	-0.03	0.00	0.07	0.05	-0.06	-0.38	0.31	0.69
ADFdig	0.17	0.33	0.14	0.22	0.32	0.26	0.24	-0.46	0.19	0.20	0.21	0.31	0.28	0.26	-0.14	0.15	0.63
STAdig	0.06	0.33	-0.03	-0.03	0.11	0.03	0.19	-0.23	0.03	0.03	0.11	0.09	0.06	0.09	-0.08	0.14	0.27
fecN excr	0.88	-0.03	0.22	0.15	0.06	-0.11	0.10	-0.37	0.85	0.85	0.62	0.78	0.78	0.79	0.57	-0.23	-0.29
urine vol	0.55	0.33	0.32	0.18	0.37	0.09	0.30	-0.55	0.58	0.58	0.48	0.60	0.52	0.56	0.31	-0.12	0.04
urineN excr	0.62	-0.04	0.23	0.18	0.13	-0.12	-0.01	-0.39	0.77	0.76	0.57	0.62	0.54	0.54	0.46	0.04	0.10
milk prodn	0.77	-0.32	0.21	0.18	-0.32	-0.49	-0.39	0.14	0.67	0.66	0.56	0.49	0.43	0.38	0.49	0.13	-0.01
milk fat	-0.24	0.27	0.10	-0.08	0.18	0.23	0.15	-0.18	-0.16	-0.15	-0.20	-0.10	-0.11	-0.17	-0.26	-0.03	0.12
milk lact	0.07	-0.07	-0.06	-0.10	-0.10	-0.11	-0.05	0.13	0.00	0.00	-0.11	-0.04	-0.02	-0.10	0.07	0.02	-0.01
MUN	-0.01	-0.14	0.14	0.10	0.13	-0.11	-0.21	0.08	0.06	0.06	0.06	0.07	-0.14	-0.13	0.03	0.08	0.08
milkN	0.79	-0.43	0.32	0.08	-0.36	-0.59	-0.45	0.16	0.71	0.68	0.53	0.47	0.42	0.49	0.54	0.13	-0.04
Nbal	0.11	0.02	0.08	0.18	0.07	0.02	0.08	-0.07	0.21	0.22	0.23	0.12	0.09	0.17	0.02	0.05	0.20
Faecal N%	-0.04	0.01	-0.25	-0.15	-0.06	0.17	0.20	-0.07	-0.20	-0.22	-0.12	-0.03	0.09	0.10	0.01	-0.40	-0.57
Urine N%	-0.44	-0.18	0.00	-0.22	0.12	-0.11	-0.18	0.29	-0.33	-0.33	-0.39	-0.34	-0.52	-0.52	-0.31	0.24	0.15
Manure N%	-0.45	-0.15	-0.12	-0.31	0.10	-0.01	-0.08	0.28	-0.43	-0.43	-0.47	-0.35	-0.47	-0.49	-0.33	0.05	-0.21
Milk N%	0.05	-0.05	-0.13	0.07	-0.42	-0.15	-0.17	0.10	-0.24	-0.25	0.07	-0.18	0.06	-0.02	0.12	0.02	-0.06
Retained N%	-0.14	-0.10	0.07	0.05	-0.04	-0.03	-0.03	0.18	-0.09	-0.04	-0.08	-0.16	-0.19	-0.14	-0.13	0.12	0.20
body weight	0.73	0.04	0.07	0.26	0.12	0.10	0.19	-0.47	0.64	0.63	0.61	0.63	0.69	0.68	0.40	-0.16	0.00

	N dig	*NDF dig*	*ADF dig*	*STA dig*	*fecN excr*	*urine vol*	*urineN excr*	*milk prodn*	*milk fat*	*milk lact*	*MUN*	*milkN*	*Nbal*	*Faecal N%*	*Urine N%*	*Manure N%*	*Milk N%*	*Retained N%*	*body weight*
Ndig	1.00																		
NDFdig	0.21	1.00																	
ADFdig	0.10	0.86	1.00																
STAdig	0.19	-0.06	-0.09	1.00															
fecN excr	-0.24	-0.14	0.13	-0.04	1.00														
urine vol	0.04	-0.06	0.18	0.21	0.49	1.00													
urineN excr	0.32	-0.11	0.01	0.03	0.54	0.64	1.00												
milk prodn	0.04	-0.19	-0.10	-0.08	0.58	0.18	0.33	1.00											
milk fat	0.02	0.28	0.28	0.07	-0.15	0.00	-0.10	-0.41	1.00										
milk lact	-0.08	-0.03	0.04	-0.03	0.03	0.06	-0.12	0.17	-0.09	1.00									
MUN	-0.02	0.05	-0.04	0.08	0.08	-0.17	0.06	0.01	-0.04	-0.13	1.00								
milkN	0.05	-0.37	-0.35	0.00	0.59	0.24	0.40	0.93	-0.36	-0.01	-0.09	1.00							
Nbal	0.30	0.14	0.18	0.00	-0.02	-0.04	-0.12	-0.07	-0.01	-0.01	0.04	-0.10	1.00						
Faecal N%	-0.90	-0.23	-0.08	-0.17	0.27	-0.09	-0.32	-0.07	0.00	0.05	0.01	-0.06	-0.45	1.00					
Urine N%	0.36	-0.05	-0.30	0.08	-0.43	-0.05	0.30	-0.12	0.03	-0.11	0.03	-0.01	-0.39	-0.25	1.00				
Manure N%	-0.11	-0.14	-0.30	0.01	-0.26	-0.12	0.10	-0.16	0.08	-0.08	0.04	-0.07	-0.63	0.31	0.84	1.00			
Milk N%	-0.28	-0.04	0.03	0.04	-0.06	-0.22	-0.38	0.45	-0.17	0.18	-0.08	0.37	-0.47	0.33	-0.31	-0.07	1.00		
Retained N%	0.27	0.15	0.11	-0.07	-0.23	-0.31	-0.28	-0.12	0.02	0.02	0.02	-0.14	0.87	-0.46	-0.33	-0.57	-0.50	1.00	
body weight	0.01	0.04	0.17	0.17	0.61	0.52	0.50	0.25	-0.10	-0.25	0.01	0.44	0.06	0.00	-0.26	-0.26	-0.09	-0.22	1.00

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