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Impact of substituting compound feed with increasing levels of fresh grass-clover on nitrogen metabolism and plasma metabolites of sows

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ABSTRACT

The purpose of this study was to identify biomarkers for fresh grass-clover intake and determine the digestibility and energy value of fresh grass to estimate voluntary fresh grass-clover intake in sows kept on pasture according to organic production practice. A total of sixteen multiparous dry sows (Danish Landrace x Danish Yorkshire) were housed in metabolism cages for 2 periods of 5 d. To study dietary effects, sows were fed one of four mixed treatments where increasing proportions of a basal commercial organic sow compound feed was partly replaced with 0, 2, 4, or 6 kg fresh grass-clover collected 3 wk after the previous cut. Sows were fed similar amounts of metabolizable energy (ME). Total collection of urine and feces was performed on a daily basis for 5d, and blood samples were collected from the jugular vein on the last day of feeding. Plasma metabolites were analyzed using a non-targeted liquid chromatography-mass spectrometry (LCMS) approach. Analyzed plasma metabolites (area under the curve in arbitrary units from the LCMS) were screened for correlation with grass intake. Data on nutrient digestibility and plasma metabolites were analyzed using a MIXED procedure while accounting for repeated measurements. Apparent total tract digestibilities of dry matter, organic matter, nitrogen, and energy of fresh grass-clover were 72, 64, 71, and 68%, respectively, using the regression method. The N intake increased linearly with increasing fresh grass-clover intake ($P < 0.001$). There was no evidence of altered N deposition and N utilization in response to increasing grass intake ($P > 0.05$). There was a linear increase in plasma urea content (2.64 to 4.39 mM) when grass intake increased from 0 to 6 kg/d ($P = 0.02$). Plasma glucose, lactate, creatinine and NEFA, and triglycerides were not affected by increased grass-clover intake. The daily ME contribution from fresh grass-clover (MJ/d) was found to be highly positively correlated with plasma pipecolic acid ($Y = 0.0289$ X; $r^2 = 0.91$; $P < 0.001$) and a metabolite tentatively identified as plasma bisnorbiotin ($Y = 0.482$ X; $r^2 = 0.92$; $P < 0.001$). In conclusion, fresh grass-clover intake of sows was highly correlated with plasma pipecolic acid and plasma bisnorbiotin concentration and apparent total tract digestibility of DM, OM, N, and energy estimated for 100% fresh grass-clover intake in dry sows was 64 to 72% using the regression method.

1. Introduction

According to the European organic regulations, organic husbandry must have access to free-range exercise or grazing, when the weather conditions permit it (Council Regulation No. 1804/1999; European Union, Brussels, Belgium). The nutritional contribution from pasture will depend on the availability, nutrient composition, intake, and digestibility of herbage, roots, and worms, and the nutritive value of foraging is thus difficult to estimate (Edwards, 2003; Blair, 2018). In Denmark, it is common practice to nose ring outdoor sows to prevent them from rooting and thereby damaging the grass sward (Eriksen et al., 2006), hence, grass species are probably the most important contributor to the daily feed intake from pasture in Danish organic sows in the grazing period from May to October. Good quality grass-clover has the potential to supply a substantial proportion of the energy, protein, and nutrients needed by organic sows from May to October (Edwards, 2003). This exacerbates the challenge in supplying a gestation- and lactation diet to match their total daily requirements for nutrients and reduce the environmental load in terms of nitrogen leaching from dietary excess. Therefore, it is of relevance to find credible methods to assess the daily intake of fresh grass-clover from pasture in outdoor sows. N-alkanes are components of the plant cuticular wax, and they have been used as markers for the estimation of grass intake and digestibility in grazing ruminants – an approach known as the n-alkane method (Mayes et al., 1986). The n-alkane method was developed for ruminants, but is also validated for

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Table 1
Dietary ingredients of the four experimental diets containing a basal organic compound feed and either 0, 2, 4, and 6 kg fresh grass-clover per day.

Item	Compound feed[1]	Compound-feed + 2 kg fresh grass- clover/d	Compound feed + 4 kg fresh grass- clover/d	Compound feed + 6 kg fresh grass- clover/d
Total ration, kg/d	2.10	3.75	5.4	7.0
Fresh grass-clover, g/kg diet	0.0	534	741	851
Compound feed, g/kg diet	1000	466	259	149
Barley	330	154	85.5	49.2
Rye	200	93.2	51.8	29.8
Oat	150	69.9	38.9	22.4
Corn	50.0	23.3	13.0	7.5
Peas	50.0	23.3	13.0	7.5
Wheat bran	35.0	16.3	9.1	5.2
Oat bran	50.0	23.3	13.0	7.5
Dried grass-clover meal	20.0	9.3	5.2	3.0
Soybean cake	47.0	21.9	12.2	7.0
Rapeseed cake	43.0	20.0	11.1	6.4
$CaCO_3$	14.0	6.5	3.6	2.1
NaCl	4.4	2.1	1.1	0.7
$Ca(H_2PO_4)_2$	6.1	2.8	1.6	0.9
Vitamin and mineral mixture[2]	1.1	0.5	0.3	0.2

[1] "Green so drægtig"; a commercial gestation diet for gestating sows, Vestjyllands Andel, Vildbjerg, Denmark
[2] Provided per kilogram of diet: 8,000 IU vitamin A; 800 IU 25-hydroxy vitamin D; 54,600 mg DL-alphatocoferol; 2,000 mg vitamin B_1; 5,000 mg vitamin B_2; 3,000 mg vitamin B_6; 20.0 mg vitamin B_{12}; 2,000 vitamin K_3; 15,000 mg D-pantothenic acid; 20,000 mg niacin; 400 biotin; 1,500 mg folic acid; 80,000 mg iron ($FeSO_4$); 15,000 mg copper ($CuSO_4$); 40,000 mg manganese (MnO); 2,000 mg iodine ($Ca(IO_3)_2$); 100,000 mg zink (ZnO); 300 mg selenium (Na_2SeO_3).

estimation of grass intake of sows (Gannon, 1996; Sehested, 1999b; Rivera Ferre et al., 2001; Kanga et al., 2012). With the n-alkane technique, it is possible to estimate the grass intake of sows without compromising the behavior or the animals´ welfare. However, it may be challenging to apply the method in outdoor pig production, since a quantification of total marker intake is essential to avoid overestimation of the grass intake. Another challenge could be fermentation. Little is known of the fate of n-alkanes in the hindgut of the sow, but some bacterial species and yeast have been shown to metabolize n-alkanes (Dostalek et al., 1968; Yamada and Yogo, 1970). It might be possible to estimate voluntary grass intake by bite-size and bite rate, short term changes in live weight, or, if the grass-clover digestibility is known, by total feces collection with a chromium marker in the compound feed. However, these techniques are both expensive, time-consuming, and challenging to apply on outdoor sows.

The aim of the present study was to develop a simple and fast method to assess the voluntary daily intake of grass-clover on pasture. We hypothesized that it is possible to identify metabolites in blood, which are linked to the intake of fresh grass-clover, and that these biomarkers can be used to predict grass-clover intake in organic sows kept on pasture. This will allow prediction of total intake of energy and dietary crude protein (CP) from fresh grass-clover in organic sows. Such knowledge may, in the future, enable optimization of the composition of organic gestation- and lactation compound feed and ultimately reduce the feed costs and N leaching from organic pig production.

2. Materials and methods

The experimental animal procedures were carried out in accordance with the Danish Ministry of Justice, Law no. 253/08.03.203 (Copenhagen, Denmark) concerning animal experiments and care and license issued by the Danish Animal Experimental Inspectorate, (Ministry of Food, Agriculture and Fisheries, the Danish Veterinary and Food Administration, Copenhagen, Denmark). The animal experiment complied with the ARRIVE guidelines (National Centre for the Replacement, Refinement and Reduction of Animals in Research, London, England) and was performed in accordance with the legislation for the protection of animals used for scientific purposes (EU Directive 2010/63/EU for Animal Experiments; Brussels, Belgium).

2.1. Animals

A total of sixteen second to sixth parity dry sows (Danish Landrace x Danish Yorkshire) with a mean live weight of 258 kg (range 191 to 320 kg) were randomly selected from the experimental herd at Aarhus University, Foulum. The sows were divided into four dietary treatments (0, 2, 4, and 6 kg fresh grass-clover/d) with four animals in each treatment. Every sow was studied during two balance periods of 5 d. During a preparation period of 3 wk, animals were stratified for willingness to consume freshly cut grass-clover, to ensure that feed allowances were within the appetite capacity of the individual animal. Some sows were consuming no or minimal amounts of fresh grass-clover in the preparation period, and these sows were allocated to treatment 1, who were supplied with only compound feed. Other sows were very eager to consume the grass-clover in the preparation period, and these sows were selected to receive 6 kg grass-clover/d. The remaining sows were distributed by chance to receive either 2 or 4 kg/d of grass-clover, respectively. There was no correlation between sow body weight or parity and the willingness to consume large amounts of fresh grass-clover in the preparation period. Individual sows remained on the same dietary treatment in the two subsequent experimental periods.

2.2. Experimental diet

A basal organic gestation compound feed based on barley, rye, oat, and rapeseed cake was formulated according to Danish recommendations to ensure recommended supply of nutrients and net energy for gestating sows (Tybirk, 2016) and purchased from a commercial supplier ("Green so drægtig"; Vestjyllands Andel, Ringkøbing, Denmark; Table 1). The four groups of sows were offered 0, 2, 4, or 6 kg freshly cut grass-clover, while the amount of compound feed was reduced concomitantly to aim at iso-energetic ME supply on a daily basis for all four groups. Prior to the experiment, the energy concentration of the grass-clover was calculated using tabulated values (Møller et al., 2000). The grass-clover was a commercial mix (ForageMax55, DLF Trifolium, Roskilde, Denmark) of 10% *Trifolium repens* (white clover, Rivendel), 50% *Lolium perenne* (perennial ryegrass, Humbi 1), 15% *Lolium perenne* (perennial ryegrass, Masai) and 25% Festuca rubra (red fescue, Gandolin). The feeding trial started October, 3rd, 2016 during a period with mild autumn climate, when the grass was 3 wk post-cut. The fresh grass was harvested every morning between 0500 and 0600 h. The

experimental diets were offered in two equally sized portions with 8h intervals during the two balance periods with total feces and urine collection. The morning diet was offered at 0800 h. Grass-clover for the afternoon feeding was stored at 5°C until supplied at 1600 h.

2.3. Housing and husbandry

At each meal and for each sow, fresh grass-clover and compound feed were weighed and distributed manually. The daily supply of freshly cut grass-clover was offered on the solid floor with side partitions to reduce lateral loss in the balance cage, and the compound feed was fed in a trough. Leftovers were collected and weighed just before the next feeding to determine the intake of feed and fresh grass-clover, which along with analyses of composition of compound feed and grass-clover, allowed estimation of the daily nutrient intake. The sows had ad libitum access to fresh water from a nipple drinker attached to a water meter. Each sow was individually housed in a metabolic cage (2.4 m × 0.8 m) made of stainless steel, with no edible bedding materials. The metabolic cages allowed a separate total collection of feces and urine, as bladder catheters were inserted at the beginning of each experimental period. Between the two collection periods, the sows remained on the same experimental dietary treatment, and sows were then group-housed for 5d in a traditional free access feeding stall with other sows receiving the same amount of grass-clover. At feeding, sows were locked inside the feeding stall for 2 h from the feeding time. Room temperature was kept at 20°C, and the light was turned on from 0700 to 1830 h. Besides the experimental recordings, the sows were managed according to the general routines in the experimental herd. Health was monitored on a daily basis by the stock personnel and weekly by the herd veterinarian.

2.4. Measurements

Sows were weighed on a walk-in scale before and after the experiment. Individual water intake was registered every day. Feed intake corrected for refusals, was recorded on a daily basis for both fresh grass-clover and compound feed. Subsamples of the compound feed were taken daily and pooled for analysis. Samples of fresh grass-clover were taken twice daily and stored at -20°C. The grass-clover was freeze-dried before analysis. Prior to the beginning of the 5d balance period, a balloon urinary catheter was inserted through the urethra into the bladder. The daily amount of urine was collected in sealed plastic jars containing 50 mL 30% H2SO4, weighed, and 10% was subsampled over the 5d for N analysis. Sulphuric acid was added to the container to prevent evaporative loss of ammonia. Total feces was collected and pooled on a weekly basis. Prior to analysis, feces were thawed, homogenized, subsampled, and freeze-dried. Blood was collected from vena Jugularis 4h after the morning feeding on d5 of each balance period. Samples were drawn in heparin vacuum tubes, placed on ice, and plasma was harvested shortly after by centrifugation at 1558 × g for 12 min at 4°C. Subsamples of plasma and fresh urine were stored at −20°C until analysis. Subsamples of plasma and fresh urine sample was stored at -80°C for metabolomics analysis.

2.5. Analytical methods

Apart from amino acids, all chemical analyses of compound feed, fresh grass-clover, feces, urine, and plasma were performed in duplicate. The dry matter (DM) content of all feed and feces samples was determined by oven drying at 103°C. Ash was determined by oven drying at 525 °C for 6h. The CP content was calculated as N × 6.25, as reported by Eggum (1991). The N content of grass, compound feed, feces, and urine was determined by the modified Kjeldahl method (Method 984.13; AOAC, 2000; KjeltecTM 2400, Foss, Hillrød, Den- lyzed in product samples hydrolyzed) or without (Arg, His, Ile, Leu, Lys, Phe, Tyr, Thr, Trp, Val) performic acid oxidation, and AA were separated by ion-exchange chromatography and quantified by photometric detection after ninhydrin reaction. Feed, grass-clover, and fecal gross energy (GE) was determined with a bomb calorimeter (Parr 6300 Instrument Company, Moline, IL, US). Starch and non-starch polysaccharides (NSP) were analyzed as described by Knudsen (1997). Plasma samples were analyzed for glucose, lactate, creatinine, non-esterified fatty acids (NEFA), triglycerides, and urea. Glucose, lactate, triglycerides, and urea were analyzed according to standard procedures (Siemens Diagnostics Clinical Methods for ADVIA 1650) on an auto-analyzer (ADVIA 1650 Chemistry System, Siemens Medical Solution, Tarrytown, NY). Plasma content of NEFA was determined using the NEFA C ACS-ACOD assay method (Wako Chemicals GmbH, Neuss, Germany). Urea and creatinine concentrations of urine were determined according to standard procedures (Siemens Diagnostics Clinical Methods for ADVIA 1650) using an autoanalyzer (ADVIA 1650 Chemistry System, Siemens Medical Solution, Tarrytown, NY). For metabolomics, plasma samples were deproteinized and prepared for analysis, as described by Soumeh et al. (2016). Freeze-dried samples (50 mg) of the compound feed, and the fresh grass-clover was mixed with 400 µL acetonitrile (10%, v/v) containing p-chlorophenylalanine and glycocholic acid (Glycine-1 13C) (0.01 mg/mL) as internal standards. The suspension was vortexed for 15 min at 4°C and centrifuged (20,800 × g, 10 min, 4°C). The supernatants were transferred to vials. Chromatographic separation of the samples was performed using a Dionex UltiMate 3000 (Dionex, Sunnyvale, CA, US) ultra-high pressure liquid chromatography system (UHPLC) equipped with an HSS T3 C18 UHPLC column, 1.8 µm, 100 × 2.1 mm (Waters Corporation, Milford, MA) equipped with a Pre-column, 100Å, 1.8 µm, 2.1 mm × 5 mm (VanGuard, Waters Corporation, Milford, MA). The column was maintained at 30°C, and the samples were placed in an autosampler kept at 10°C during the entire run. The mobile phases were 0.1% formic acid in Milli-Q water (A) and 0.1% formic acid in acetonitrile (B). The flow rate was 0.4 mL/min. The gradient program was as follows: 0 to 12 min, linear gradient from 5 to 90% B; 12 to 12.3 min, 90% B, and return to initial conditions in 0.2 min. Corresponding changes in A were made. The column was re-equilibrated at 5% B for two min at the beginning of each run. The eluent was introduced into an Ultra-High Resolution mass spectrometer (Qq-Time-Of-Flight, Impact HD, Bruker Daltonics GmbH, Bremen, Germany) by electrospray ionization and analyzed in positive and negative ionization modes using the instrumental parameters described by Hedemann (2017). To evaluate the analytical performance of the system, blank samples (5% acetonitrile) and quality controls (QC, a pooled plasma sample) were analyzed after each five or 10 samples, respectively, to check for potential cross-contamination from samples and system reproducibility during the run.

2.6. Calculations

The 24 h losses of energy via feces and urine were quantified using total collection. Undigested DM was calculated from average daily feed intake (ADFI) and DM apparent total tract digestibility (ATTD), and the fecal GE output was then quantified by multiplying undigested DM with the analyzed GE concentration in feces. The ATTD was calculated using both the regression and difference methods. In the identification of metabolites from fresh grass-clover, mass spectra were calibrated and converted to mzXML-spectra using CompassXport 3.0.9.2 (Bruker Daltonics GmBH, Bremen, Germany) and preprocessed using an R-based XCMS software package (Smith et al., 2006). The exported data tables were filtered to eliminate features present in blanks, retention times were truncated to contain only portions with chromatographic peaks, and masses higher than 800 m/z were discarded (m/z represents mass to charge ratio). The data tables were imported to LatentiX 2.10 (Latent5 Aps, Gilleleje, Denmark) and were Pareto-scaled, which reduces the importance of high-intensity peaks but retains the variability in the data structure partially intact (van den Berg et al., 2006) then

Principal Component Analysis (PCA) was performed. Loadings plots were used to identify metabolites discriminating sows eating 0 kg grass from sows eating 6 kg grass, and plots were made to check for linear correlation between intake of grass and the level of the metabolite in plasma. The relevant metabolites were identified using accurate masses and fragmentation patterns based on searches in online databases: the METLIN (http://metlin.scripps.edu/), Human Metabolome Database (http://www.hmdb.ca/), and LIPID MAPS (http://www.lipidmaps.org/). The identification of the annotated compounds was confirmed with standards, when available, on the same analytical system under the same conditions (validation based on retention time and mass spectra).

2.7. Statistical analysis

The statistical analysis was performed using the MIXED procedure of SAS (Ver. 9.4; SAS Inst. Inc., Cary, NC, US) with the following statistical model:

$Y_{ijk} = \mu + \alpha_i + \beta_j + \alpha\beta_{ij} + \nu_k + \varepsilon_{ijk}$, in which Y_{ijkl} is the response variable, μ is the overall mean, α_i is the fixed effect of dietary treatment (i = 1,…, 4), β_j is the effect of period (j = 1, 2), $\alpha\beta_{ij}$ is the interaction term between dietary treatment and period, ν_k is the random effect of the k'th sow (k = 1, 2, …,16), and ε_{ijkl} is the random error component which was assumed to be N (0, σ2).

Plasma NEFA was subjected to a logarithmic transformation to stabilize the residual variance. All variables were considered significant when $P < 0.05$ and tendencies were accepted at $P \leq 0.12$. Mean values are represented as least squares means ± standard error of the mean (pooled SEM) or back-transformed mean with lower and upper 95% confidence intervals. The Tukey-Kramer test was used in the ANOVA test to adjust the P-values in multiple comparisons of means. In addition, orthogonal polynomial contrasts were used to evaluate linear and quadratic effects of grass-clover intake.

3. Results

Two sows (supplied with 2 and 4 kg fresh grass-clover, respectively) were excluded from all analyses, as they developed fever and had substantial feed and grass-clover reidues. Apart from these sows, all sows in generally consumed their full rations of grass-clover, and sows that were offered 2, 4 and 6 kg of fress grass-clover per day consumed on average 1.91, 4.00 and 6.00 kg/d, respectively.

3.1. Chemical composition of fresh grass-clover

Increasing the allocation of grass from 0 to 6 kg/d increased the CP content of the total diet from 129 to 209 g/kg DM (Table 2). The Lys concentration in the total mixed diet increased from 6.3 g/kg DM in the diet without grass-clover to 11.1 g/kg DM in the diet including 6 kg grass-clover/d. Methionine increased from 2.0 to 3.3 g/kg DM, and threonine from 4.7 to 8.7 g/kg DM in the rations with 0 and 6 kg grass-clover/d, respectively. Cysteine was fairly constant across the dietary rations, whereas the remaining AA in the total rations also increased gradually with increasing levels of grass-clover. The AA accounted for 92 and 85% of the dietary CP in the 0 and 6 kg total diets, respectively.

3.2. Voluntary grass intake

Three sows lost weight during the experiment (average -2.4 kg). The remaining eleven sows had a minor weight gain (average +4.4 kg; Table 3). The individual weight changes were not affected by grass intake. The fresh grass-clover contained 16% DM, which was 3%-units less than expected (19%) in the dietary formulation. Unintendedly, the intake of ME therefore decreased linearly from 25.7 to 22.1 MJ/d ($P <$ of fresh grass-clover. There was no ater intake or feces output.

3.3. Digestibility of fresh grass-clover for sows

The apparent total tract digestibility (ATTD) of energy in the total diet declined linearly from 80.4 to 73.6% with increasing grass-clover inclusion ($P < 0.05$; Table 4). The ATTD of organic matter (OM) was reduced from 79.8 to 72.6% ($P < 0.05$). There was no effect of increasing grass inclusion on ATTD N in this study. The ATTD DM tended to decrease with increasing grass-clover intake ($P = 0.09$). Using the regression method, the ATTD of DM, OM, N and energy of 100% grass-clover were 70.0 ± 3.4%, 67.0 ± 3.5%, 74.7 ± 2.7% and 68.7 ± 3.4%, respectively. Using the difference method, DM digestibility of 100% fresh grass-clover ranged between 68.6 to 72.2%, digestibility of OM of grass ranged between 58.9 to 62.9%, digestibility of N of grass-clover was between 70.4 to 79.9%, and digestibility of grass-clover ranged between 67.4 to 69.5%.

3.4. Biomarkers for grass-clover intake

The PCA-scores plot of plasma in positive mode showed a clear separation between sows fed no grass-clover and sows fed 6 kg/d of grass-clover, and especially principal component two was high when sows did not consume any grass and decreased with increasing grass intake (Fig. 1). Inspecting the loadings plot and correlating the area of the peaks to the intake of grass-clover showed that three metabolites important for the discrimination between the dietary treatments correlated to intake of grass. The metabolites were identified as pipecolic acid (m/z 130.0864), and a fragment of pipecolic acid (m/z 84.0808) and m/z 217.0686 was tentatively identified as bisnorbiotin. Inspecting the chromatograms of grass-clover and the compound feed showed that fresh grass-clover contained a clear peak of pipecolic acid, whereas the compound feed only showed a minor peak (Fig. 2). Searching the chromatograms for m/z 217.0686 showed a minor peak in the compound feed, whereas no peak was found in grass-clover. The daily ME (MJ/d) contribution from fresh grass-clover was found to be highly positively correlated with plasma pipecolic acid (Y = 0.0289 X (SE = 0.0011); R^2 = 0.91; $P < 0.001$) and plasma bisnorbiotin (Y = 0.482 X (SE = 0.019); R^2 = 0.92; $P < 0.001$; Fig. 3). The daily intake of digestible OM from grass-clover in g/d may be predicted as Y = 0.871 X (SE = 0.033); R^2 = 0.91, where X is plasma pipecolic acid or Y = 14.5 X (SE = 0.7); R^2 = 0.89, where X is the metabolite tentatively identified as plasma bisnorbiotin.

3.5. Plasma metabolites

Plasma concentrations of urea ($P = 0.04$), pipecolic acid and bisnorbiotin ($P < 0.001$) increased linearly when grass intake increased from 0 to 6 kg/d (Table 5). Plasma creatinine decreased with increasing grass intake in a curvilinear manner. Moreover, a tendency for a quadratic effect was observed on NEFA with the highest concentrations observed in sows fed 0 or 6 kg/d and lowest in sows fed 2 kg/d of grass. There was no effect of fresh clover grass intake on plasma lactate, glucose, or triglycerides.

3.6. Nitrogen balance

The N intake increased linearly with increasing fresh grass-clover intake ($P < 0.001$; Table 6). The N excretion in urine and feces in g/d increased with increasing grass-clover intake ($P < 0.003$). The N retention was not affected by grass intake within the range investigated in this experiment, neither when expressed in g/d nor in % of intake.

4. Discussion

4.1. Voluntary grass-clover intake

There was a large between-animal variation in voluntary grass-

Table 2
Analyzed composition of fresh grass-clover and basal gestation diet (0 kg grass-clover diet) and calculated chemical composition of the three diets containing fresh grass-clover.

Chemical composition, g/kg DM	Fresh grass- clover[1]	Compound feed[1]	Compound feed + 2 kg fresh grass-clover/d[2]	Compound feed + 4 kg fresh grass-clover/d[2]	Compound feed + 6 kg fresh grass-clover/d[2]
Dry matter, g/kg feed	163	877	496	348	270
Protein (Nx6.25)	284	129	156	183	209
Fat	-	38.0	-	-	-
Starch	17.9	482	401	321	243
Cellulose	121	49.0	61.6	73.9	85.9
Total NSP[3]	282	176	194	212	230
Insoluble NSP[3]	212	148	159	170	181
Klason lignin	77.5	57.0	60.6	64.1	67.6
Ash	131	48.0	62.7	77.0	90.9
Dietary fiber	359	233	255	276	298
Gross energy, MJ/kg DM	18.5	17.9	18.0	18.1	18.2
Amino acids, g/kg DM					
Lys	15.57	6.32	7.94	9.53	11.08
Met	4.47	2.00	2.43	2.85	3.27
Cys	2.56	2.68	2.66	2.64	2.62
Val	15.86	6.18	7.88	9.54	11.17

[1] Analyzed chemical composition. This composition represents 0 kg/d of grass-clover intake.
[2] Calculated from the analyses and intake of grass-clover and compound feed (assuming no feed residues).
[3] Non-Starch Polysaccarides

clover intake during the 3 wk of training period. The animals were housed indoor and had never experienced fresh grass before being included in this experiment. A few sows in the adaptation period would not ingest any grass at all and were therefore assigned to the total diet without grass-clover. This grass rejection by some sows was also reported in another experiment (Rivera Ferre, 1999). Edwards (2002) suggested that the daily herbage intake of sows ranges from 2 to 10 kg/d with an average of 6 to 7 kg/d. Gannon (1996) reports individual variations in herbage intake in the range between 1.1 and 10.5 kg/d of fresh herbage in the spring, and 4.3 to 11.8 kg/d in the summer. Therefore, the contribution of grass-clover to the daily nutrient intake, and the degree to which grass-clover can substitute a compound feed, might also vary greatly among individuals within a herd. We saw no relationship between sow weight and tendency to voluntarily consume 6 kg of fresh grass-clover.

The contribution of energy and CP from the compound feed might affect the grass-clover intake of the sows, but in this experiment, there were no grass-clover residuals, suggesting the daily upper limit might be higher than 6 kg/d of fresh grass-clover. Using live weight measurements (Fernandez, 2006) also found no effect of allocated concentrate on the intake of grass-clover in 15 pregnant sows on pasture in May-June and August-September. Gaseous energy losses, i.e., methane emission, was not considered when calculating dietary ME in the present study, and may marginally cause an overestimation of the reported ME values.

4.2. Biomarkers for voluntary grass intake

Plasma piperidine-2-carboxylic acid, more commonly known as pipecolic acid, increased linearly with increasing DM from fresh grass-clover. L-pipecolic acid is identified as a constituent of legumes (Broquist, 1991) and Morrison (1953) isolated pipecolate (500 mg) from 500 g of cut leaves of white clover; hence it is likely that the increase in plasma pipecolic acid shown in this experiment, derives from the 10% white clover in the grass-clover mix. However, we also found a very small amount of pipecolic acid in plasma in sows fed the

Table 3
Intake and utilization of energy, urine and feces production in dry sows fed increasing amounts of freshly cut grass-clover and iso-energetic diets.

		Daily fresh grass-clover supply, kg *P*-value					*P*-value		
Item		0	2	4	6	SEM[1]	ANOVA	Linear	Quadratic
No. of sows	4	3	3	4					
Start body weight, kg	275	249	252	255	20	0.73	0.53	0.45	
Body weight change, kg	2.6	2.1	0.3	2.9	1.9	0.74	0.91	0.37	
Total GE[2] intake, MJ/d		33.3	33.5	34.3	34.1	0.3	0.04	0.01	0.46
Total DE[2] intake, MJ/d		26.7	26.3	25.7	23.0	0.9	0.10	0.02	0.27
Total ME[2] intake, MJ/d		25.7	25.3	24.7	22.1	1.0	0.10	0.02	0.28
DM intake compound feed, kg/d	1.86^{a}	1.55^{b}	1.24^{c}	0.88^{d}	0.08	<0.001	<0.001	0.43	
DM intake grass-clover, kg/d		0.0^{d}	0.31^{c}	0.66^{b}	0.99^{a}	0.01	<0.001	<0.001	0.38
Dig. DM[3] intake from grass-clover kg/d	0^{d}	0.21^{c}	0.45^{b}	0.71^{a}	0.01	<0.001	<0.001	0.03	
GE[2] intake from grass-clover, MJ/d	0^{d}	5.7^{c}	12.1^{b}	18.3^{a}	0.2	<0.001	<0.001	0.41	
DE[2] intake from grass-clover, MJ/d	0^{d}	4.0^{c}	8.5^{b}	12.3^{a}	0.2	<0.001	<0.001	0.01	
ME[2] intake from grass-clover, MJ/d	0^{d}	3.8^{c}	8.2^{b}	11.8^{a}	0.2	<0.001	<0.001	0.67	
Water intake, l/day	18.9^{ab}	23.4^{a}	9.11^{b}	14.3^{ab}	3.3	0.05	0.05	0.91	
Urine, kg/day		7.6^{ab}	5.2^{b}	5.7^{b}	10.3^{a}	1.1	0.04	0.11	0.01
Feces, g DM/day		396^{b}	455^{ab}	447^{ab}	578^{a}	330	0.21	0.26	0.04

[a–d] Within a row, values without a common superscript differ ($P < 0.05$).
[1] SEM = standard error of the means.
tible and metabolisable energy, respectively.

Table 4
Apparent total tract digestibility (ATTD) of dry matter (DM), organic matter (OM), nitrogen and gross energy of dry sows fed varying amounts of freshly cut grass-clover and iso-energetic diets.

	Daily fresh grass-clover supply, kg					*P*-value		
ATTD, %	0	2	4	6	SEM[1]	ANOVA	Linear	Quadratic
DM (total ration)	80.8	78.0	75.5	74.7	1.7	0.09	0.02	0.59
OM (total ration)	79.8	76.5	73.5	72.6	2.0	0.05	0.008	0.53
Nitrogen (total ration)	81.0	79.0	77.6	78.6	1.2	0.47	0.24	0.34
Energy (total ration)	80.4	78.3	74.5	73.6	2.0	0.05	0.008	0.77

[1] SEM = standard error of the means.

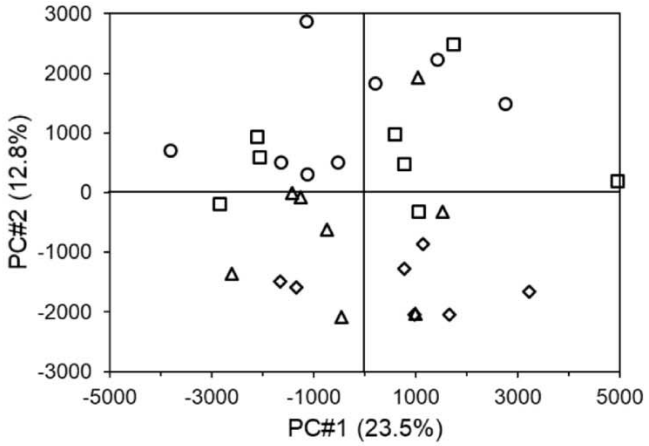

Fig. 1. Principal Component Analysis scores plot showing clustering of the sow plasma metabolome by amount of grass-clover consumed (0, 2, 4, or 6 kg of grass-clover/d) using an unsupervised method in positive ionisation mode (Fresh grass-clover intake: *o* = 0 kg/d; □ = 2 kg/d; Δ= 4 kg/d; ◆ = 6 kg/d). The variances accounted for by the principle components are shown on the axes.

total diet without any grass-clover. Pipecolic acid is an AA first identified in plasma of cows and goats and Onodera and Kandatsu (1969) showed that pipecolic acid was produced from L-lysine by rumen ciliate protozoa but not by rumen bacteria. Chavatte et al. (2016) demonstrated that protozoa are common in the gastrointestinal tract of pigs. Therefore, Lys intake from the compound feed can explain the intercept in plasma pipecolic acid, and it seems likely that the small amount of pipecolic acid in plasma from sows fed the total diet without grass-clover most likely was produced by protozoa in the hindgut of the sows. The metabolite tentatively identified as plasma bisnorbiotin, increased linearly like pipecolic acid with increasing grass-clover intake. Bisnorbiotin is a major biotin metabolite and has been detected in pig plasma after intravenous administration of physiologic amounts of biotin (Wang et al., 2001). Biotin is synthesized from cellulose by microbial fermentation in the hindgut. Since the daily intake of cellulose was increased by 75% when increasing grass supply from 0 to 6 kg/d of grass-clover, this likely explains the highly positive correlation between plasma bisnorbiotin and fresh grass-clover intake. There is one argument saying that plasma bisnorbiotin might be a more applicable marker for grass-clover intake in organic sows than pipecolic acid since there was no intercept (i.e., no bisnorbiotin at 0 kg grass-clover). On the other hand, there was a very high content of pipecolic acid in the grass-clover but not in the compound feed, and the metabolite tentatively identified as bisnorbiotin is found in a much lower concentration in plasma than pipecolic acid, which makes pipecolic acid a reliable marker for fresh grass-clover intake. It is possible that the relative plasma concentrations of pipecolic acid and bisnorbiotin may vary with [illegible] state of the pigs due to varying con[illegible]tion in the digestive tract and with number of weeks post cut. Different grass varieties differ greatly in the relative proportion of CP and carbohydrates, and it also seems likely that changes in Lys and cellulose content during the growing season of grass-clover may affect plasma concentrations of the studied metabolites in pigs. The validity of grass intake estimates by using plasma bisnorbiotin or pipecolic acid as a marker might also be affected by the composition of the supplementary diet, and ingredients such as grass meal containing clover must be avoided when formulating the supplementary diet. Further investigations are needed to evaluate how robust these metabolites are, when predicting grass-clover intake.

4.3. Digestibility of fresh grass-clover for sows

The ATTD of energy in the total diet decreased from 80.4 to 73.6%, when fresh grass-clover intake increased from 0 to 6 kg/d. This is also seen in other studies, as dietary fiber is associated with impaired nutrient utilization and reduced net energy values (Lindberg, 2014), and the level of dietary fiber can linearly reduce the ATTD of DM, OM, energy, CP, and non-fibrous carbohydrates in sows (Noblet and Le Goff, 2001; Oelke et al., 2018). The ATTD of OM in the total diet was also affected by the level of grass inclusion ($P < 0.05$). The ATTD of grass-clover diet was estimated using both the regression and the difference method. The energy digestibilities around 68% indicate, that the grass was fairly digestible, which corresponds well with Carlson et al. (1999), who found total energy digestibilities of a total diet with compound feed and freshly cut grass-clover (18% of DM) to be 82% in 30-kilo gilts. The fresh grass-clover constituted 53% of DM in the total diet with the greatest inclusion level of grass-clover in our study. The digestibility of N in the total diet appeared not to be affected by the inclusion level of fresh grass-clover within the studied range.

4.4. Plasma metabolites

All plasma glucose concentrations were within the normal range of pigs, but there was a tendency to decreased plasma glucose concentrations with increasing grass-clover intake ($P = 0.11$). This is in accordance with (de Leeuw et al., 2004) who found that fermentable dietary fiber stabilized interprandial blood glucose levels in pregnant sows, and Serena et al. (2009) who finds lower net absorption of glucose 2h after feeding in multi-catheterized sows fed high fiber diets as compared with a low-fiber diet. The dietary effect on NEFA was probably also related to the shift in energy being net absorbed from the small intestine (mainly as glucose) to hindgut fermentation (mainly uptake of short-chain fatty acids) when comparing sows without any grass intake with the sows receiving substantial amounts of grass. It should be emphasized that the blood samples in this study were taken approximately 4h after feeding and thus represent a fed state, although most of the glucose from digested starch is already net absorbed at this time (Serena et al., 2009). Most likely, this pattern would not be consistent throughout the day because fluctuations in net energy absorption is much more pronounced when fiber and roughage intake is low.

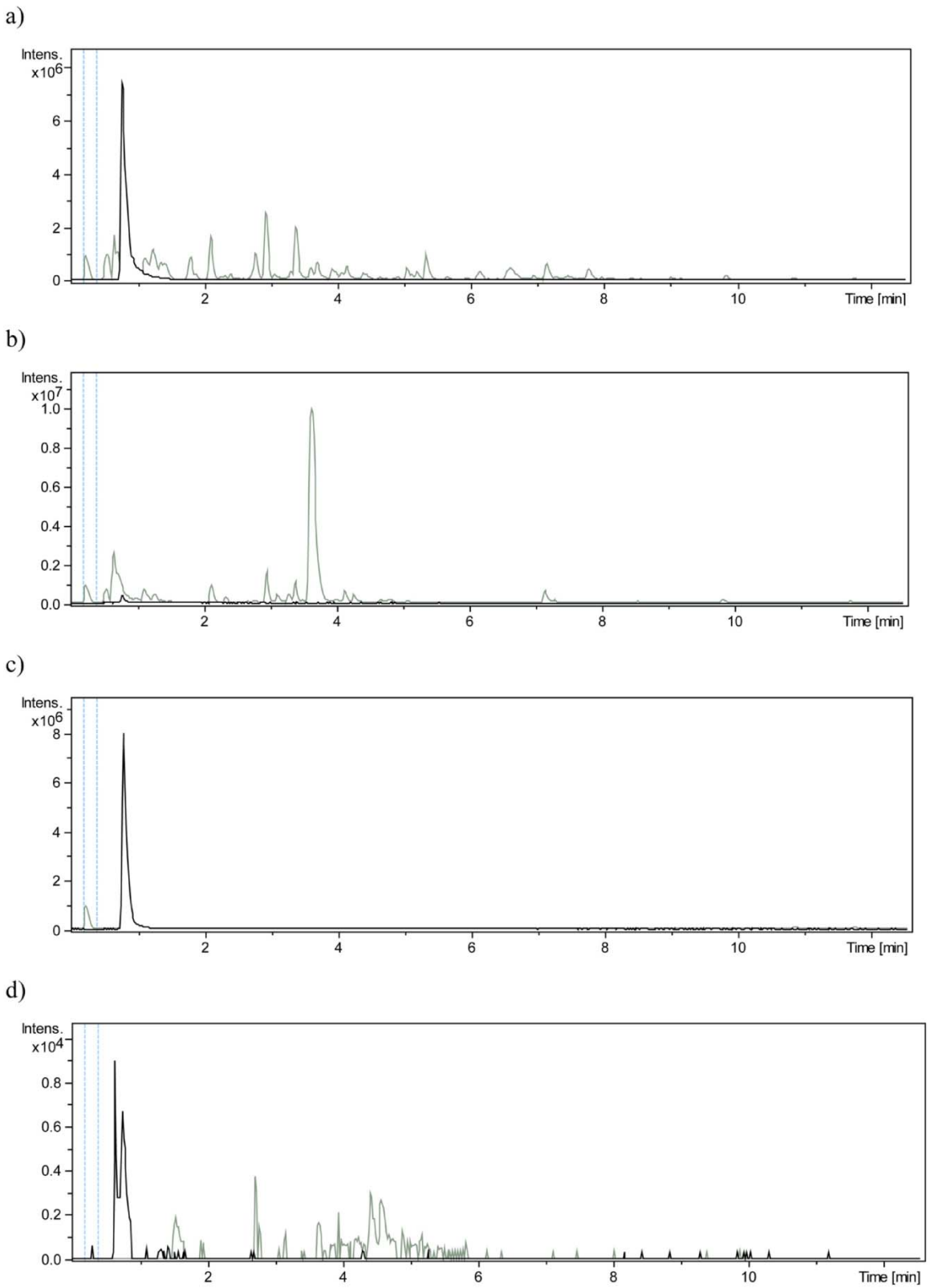

Fig. 2. Representative base peak chromatograms (grey) with extracted ion chromatograms of m/z 130.0862 (black) of a) grass-clover (3 wk post-cut), b) compound feed, and c) synthetic pipecolic acid (Sigma-Aldrich P2519, Merck, Darmstadt Germany) and d) extracted ion chromatogram of m/z 217.0686, tentatively identified as bisnorbiotin, in the compound feed (black) and grass-clover (grey) using LC/ESI-QTOFMS (Liquid chromatography electrospray ionization Qq-Time-Of-Flight mass spectrometry) in positive ionization mode.

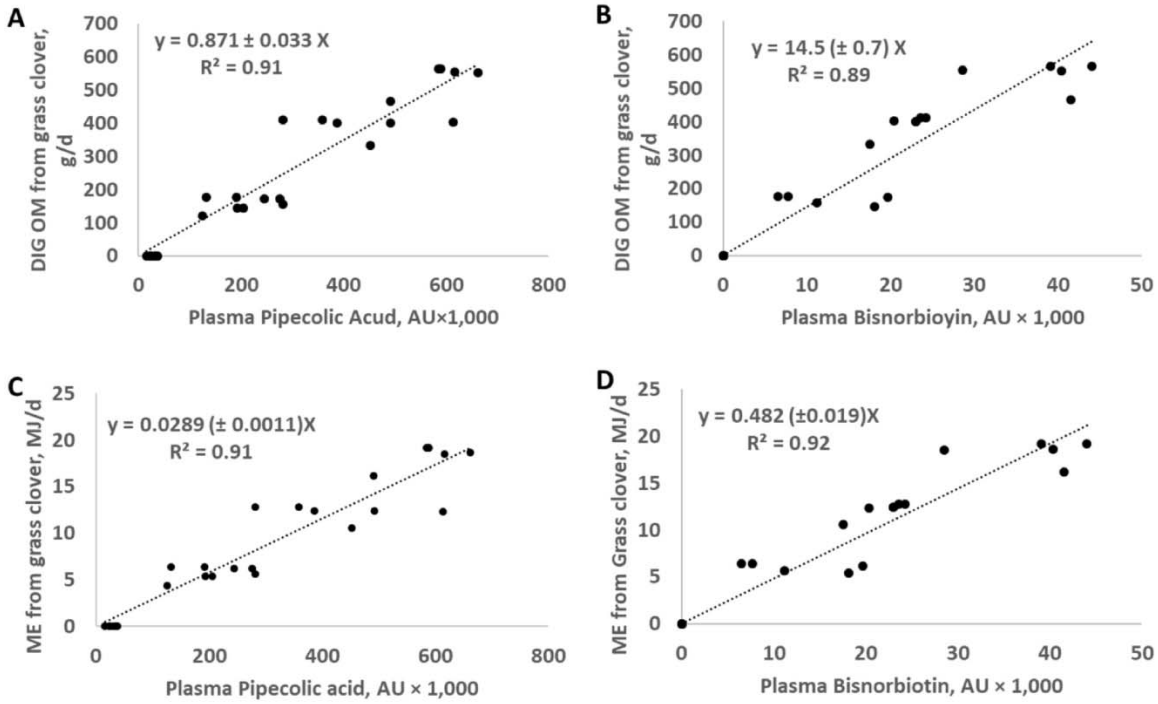

Fig. 3. Apparent total tract digestible organic matter (Dig. OM; panel A and B) and metabolizable energy (ME; panel C and D) intake from fresh grass-clover/d as a function of plasma pipecolic acid or plasma bisnorbiotin, respectively, in dry sows fed increasing fresh grass-clover and decreasing compound feed.

4.5. Nitrogen balance

As the CP content in the diet increased with increasing allocation of fresh grass-clover, there was an increase in N intake from 43.6 g/d to 65.2 g/d. Grass-clover substitution caused an oversupply of all essential AA compared to the nutrient recommendations (Tybirk, 2016). Urinary N losses were 27.3 g/d in sows fed the total diet without grass-clover whereas it increased linearly to 48.4 g/d in sows fed the total diet with greatest inclusion level of grass-clover. As this cannot be explained by a less optimal AA profile in fresh grass-clover (5.5 g Lys/16 g N) as compared with the corn-based compound feed (4.9 g Lys/16 g N), it is most likely due to the greater N intake in sows fed increasing levels of grass-clover in the total diet. Plasma urea increased linearly with increasing grass intake, indicating that sows were supplied with excess CP when grass replaced the compound feed. A negative consequence of CP oversupply is that metabolism and excretion of excessive CP reduces the energy utilization by up to 6% (Pedersen et al., 2019). Hence, sows will require more feed to reach the same productivity level, if the contribution of CP from grass is not balanced relative to the intake of net energy in the total diet for sows on pasture. Increasing fresh grass-clover inclusion in the diet doubled the fecal N output. Increased fecal N output was also reported by Lindberg et al. (1995) and Vestergaard (1996), who increased forage meal inclusion in a barley-based diet for finishing pigs. The increased grass-clover intake did neither affect N deposition in g/d nor when expressed in percent of intake in our study. Oversupply with CP and AA is seen to result in an increased N content in the slurry and consequently increased environmental pollution due to N emission and leaching (Nørgaard et al., 2014).

5. Conclusion

Increasing grass-clover intake linearly increased the daily CP intake but decreased the apparent total digestibility of dry matter, organic matter, N, and energy. The daily ME contribution from fresh grass-clover was found to be highly positively correlated with plasma pipecolic acid and plasma bisnorbiotin. The present data confirmed that fresh grass-clover is a good source of both CP and energy in organic sow

Table 5
Plasma metabolites in dry sows fed varying amounts of fresh grass-clover and iso-energetic diets.

Plasma metabolites	Daily fresh grass-clover supply, kg					*P*-value		
	0	2	4	6	SEM[2]	ANOVA	Linear	Quadratic
Urea, mM	2.64[b]	4.05[ab]	3.59[ab]	4.39[a]	0.38	0.04	0.02	0.42
Glukose, mM	4.52	4.45	4.27	4.16	0.15	0.39	0.11	0.91
Lactate, mM	2.03	1.26	1.35	1.89	0.47	0.55	0.87	0.18
Creatinine, µM	159	143	148	125	12	0.26	0.26	0.02
NEFA[3], µM	81.8	33.5	37.4	84.8	27.0	0.39	0.92	0.09
Triglycerides, µM	222	251	298	285	27	0.20	0.06	0.44
Pipecolic acid, AU[1]	28.3[d]	206.2[c]	435.8[b]	600.0[a]	31.0	<0.001	<0.001	0.83
Bisnorbiotin, AU[1]	0.0[d]	12.1[c]	21.9[b]	38.8[a]	2.3	<0.001	<0.001	0.88

[a–d] Within a row, values without a common superscript differ ($P < 0.05$).
[1] Arbitrary Units x 1,000

Table 6
Nitrogen balance and urinary concentrations of creatinine and urea in dry sows fed varying amounts of fresh grass-clover and iso-energetic diets.

	Daily fresh grass-clover supply, kg					*P*-value		
Item	0	2	4	6	SEM[1]	ANOVA	Linear	Quadratic
N intake, g/d	43.6[d]	50.3[c]	58.5[b]	65.2[a]	3.8	<0.001	<0.001	0.93
N in feces, g/d	8.3[b]	10.4[b]	12.7[ab]	16.8[a]	1.5	0.003	0.003	0.49
N in urine, g/d	27.3[b]	33.0[b]	34.1[b]	48.4[a]	2.6	<0.001	0.001	0.49
N retention, g/d	6.5	6.9	9.7	0.3	2.7	0.47	0.46	0.23
Creatinine in urine, mM	8.02[ab]	10.04[a]	9.41[ab]	3.43[b]	1.46	0.05	0.06	0.02
Urea in urine, mM	75.3	142.0	130.4	69.3	25.8	0.18	0.80	0.04
N retention, % of intake	14.9	13.9	16.7	2.8	6.2	0.43	0.24	0.30

[a–d] Within a row, values without a common superscript differ ($P < 0.05$).
[1] SEM = standard error of the means.

production. To avoid CP oversupply and N leaching, it is important to counterbalance the N contribution from fresh grass-clover when formulating compound feed for grazing sows.

CRediT authorship contribution statement

Maria Eskildsen: Methodology, Formal analysis, Investigation, Data curation, Writing - original draft, Writing - review & editing, Visualization. **Mette Skou Hedemann:** Methodology, Validation. **Peter Kappel Theil:** Conceptualization, Supervision, Writing - review & editing, Project administration, Funding acquisition. **Jan Værum Nørgaard:** Methodology, Formal analysis, Supervision.

Declaration of Competing Interest

All authors declare that they have no conflicts of interest.

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