Sheep urination frequency, volume, N excretion and chemical composition: implications for subsequent agricultural N losses

Karina A. Marsden*[a,b], Lucy Lush[c], Jon. A. Holmberg[a], Mick J. Whelan[d], Andrew J. King[c,e], Rory P. Wilson[c], Alice F. Charteris[f], Laura M. Cardenas[f], Davey L. Jones[a,g], David R. Chadwick[a]

[a] *School of Natural Sciences, Bangor University, Bangor, Gwynedd, LL57 2UW, UK*

[b] *Faculty of Veterinary and Agricultural Sciences, The University of Melbourne, Parkville, Victoria, 3010, Australia*

[c] *Department of Biosciences, College of Science, Swansea University, Singleton Park, Swansea, SA2 8PP, UK*

[d] *Centre for Landscape & Climate Research, University of Leicester, Geography, Leicester, LE1 7RH, UK*

[e] *Institute for Communities and Wildlife in Africa, Department of Biological Sciences, University of Cape Town, Cape Town, South Africa*

[f] *Rothamsted Research, Sustainable Agriculture Sciences, North Wyke, Okehampton, Devon, EX20 2SB, UK*

[g] *UWA School of Agriculture and Environment, The University of Western Australia, Perth, WA 6009, Australia*

*Author for correspondence:

Tel: +441248 383052

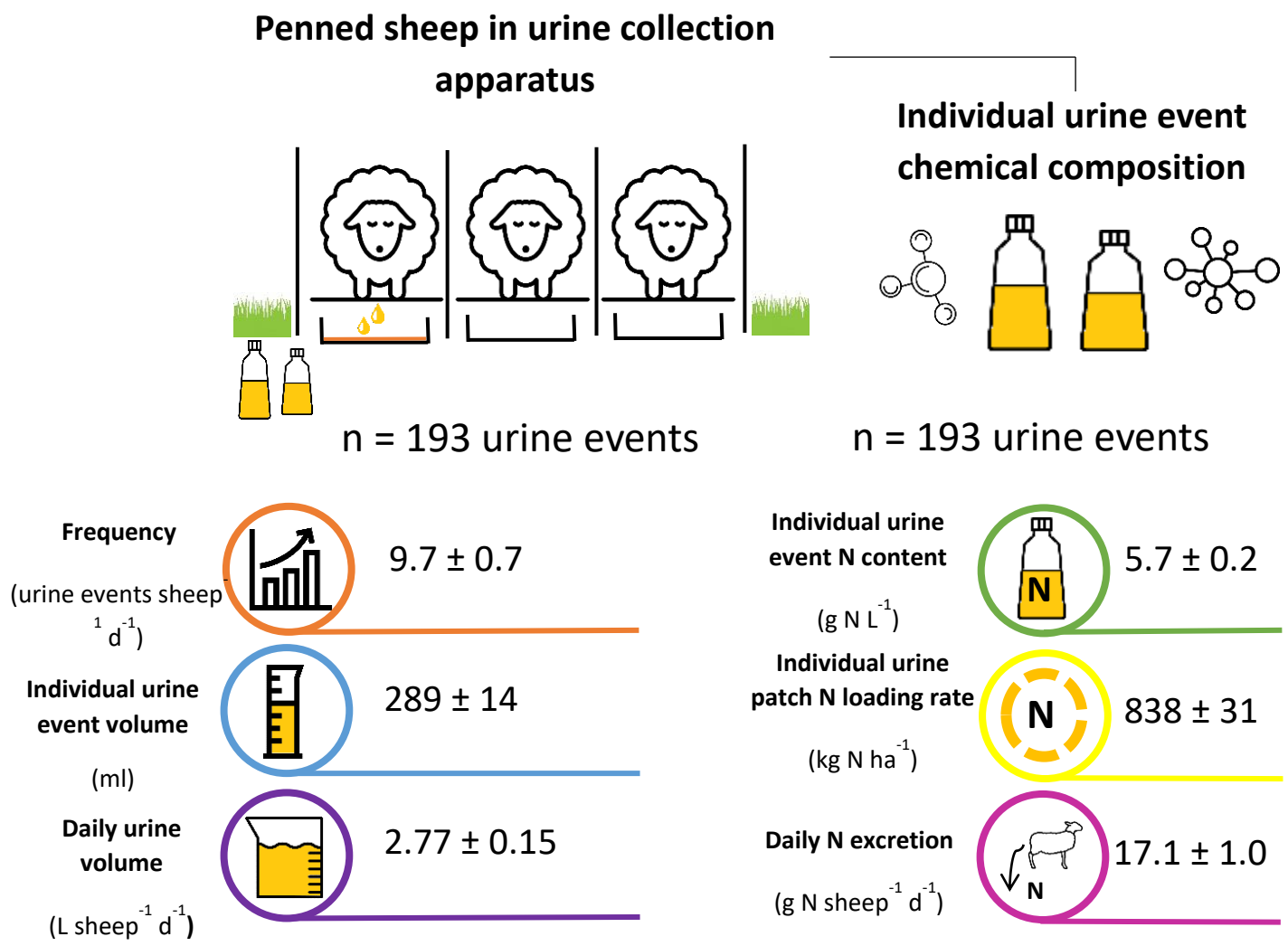
Email: k.marsden@bangor.ac.uk

Abstract

Ruminant urine patches are potential sites of reactive nitrogen (N) loss to the environment. Quantification of N losses from grazed grasslands requires measurement of the frequency of urine deposition, as well as its volume and chemical composition. However, studies to date are typically restricted to analyses of few replicate animals and urination events, especially for sheep. Here, we present data on urine frequency, volume, chemical composition (n = 193 events from n = 6 sheep) and metabolomic profile (n = 4 - 5 events from n = 4 - 5 sheep) from penned sheep. Differences in urine parameters and chemical composition data were compared seasonally and between two sites (improved and semi-improved pasture). Sheep urinated 8 to 11 times d^{-1}, assuming time within pens represented a 24 h period. The mean urine event volume recorded was 289 ± 14 mL, from which we estimated a daily urine production value of 2.77 ± 0.15 L urine $sheep^{-1}$ d^{-1}. Daily urine N excretion and individual urine N concentrations were greater from sheep in improved pasture (26.7 ± 2.3 g N $sheep^{-1}$ d^{-1}; 7.0 ± 0.2 g N L^{-1}) compared to those in semi-improved pasture (16.7 ± 1.2 g N $sheep^{-1}$ d^{-1}; 5.5 ± 0.4 g N L^{-1}), but this did not equate to greater individual urine patch N loadings due to site differences in the urine-to-soil surface area influenced (17.5 L m^{-2} at the semi-improved site and 8.9 L m^{-2} at the improved site). Urine chemical composition varied seasonally and by site. Site- and season-specific urine should, therefore, be used in studies assessing N losses from urine patches. Based on the urine chemical composition data, we provide an updated artificial sheep urine 'recipe' which could be utilised to replicate natural sheep urine. The urine metabolomic profile clustered according to pasture quality, while clustering according to season was less evident. Our results provide important information for experimental and modelling studies assessing the scale and nature of N pollution arising from sheep-grazed pastures.

Key Words: Nitrogen cycle; Ruminant; Grazing; Livestock; Excreta; Metabolome

Graphical Abstract



Icons sourced from the Noun Project (https://thenounproject.com/), sheep by Vectors, droplet by Alex Muarvev, washing liquid by Made by Made, bar chart by Shastry, graduated cylinder by Georgiana Ionescu, beaker by iconix and chemicals by ibrandify.

1. Introduction

The urine patches of grazing animals are well recognised hotspots of nitrogen (N) losses to the environment, including ammonia (NH_3) volatilisation, nitrate (NO_3^-) leaching and the transfer of nitrogen oxides (NO_x), nitrous oxide (N_2O) and nitrogen gas (N_2) from the soil to the atmosphere (Clough et al., 2003; Zaman and Nguyen, 2012; Harrison-Kirk et al., 2015). Each of these losses has potential environmental and/or economic implications, including off-site soil acidification (Goulding et al., 1998), eutrophication of receiving water bodies (Fenn et al., 1998), increases in greenhouse gas concentrations (Lashof and Ahuja, 1990) and the indirect catalysis of stratospheric ozone depletion (Ravishankara et al., 2009). In the case of N_2 its emissions represent an economic loss for the farmer. At the individual urine patch scale, the fate of urine-N is linked to the frequency of urination events, urine volume, its chemical and N composition (Hoogendoorn et al., 2010) and to soil conditions (van Groenigen et al., 2005).

Datasets on such urination parameters, are rare and tend to be small in size, particularly for sheep (e.g. number of collected urine events or number of individual animals used; see Selbie et al. (2015) for a meta-analysis of recently published information on ruminants). Furthermore, they are often of limited use as they do not include all the meta-data/information needed for assessing associated up-scaled environmental pollution. Of the available data, variability has been shown to be high at the individual animal level, between grazing species (e.g. cattle vs. sheep; Hoogendoorn et al., 2010) and diurnally (Minson and Cowper, 1966). There is, therefore, a need to increase the number and improve the quality of available datasets on urine patch parameters, the number of constituent replicate animals and/or urine events and the number of recorded urine patch parameters to better predict subsequent N losses.

In addition, variability exists at the individual urine patch scale e.g. individual urine event volumes and N concentrations interact to produce patches with highly variable N loading rates.

This, in turn, leads to spatially and temporally variable N loading rates and associated environmental pollution. For example, Selbie et al. (2014) found a diminishing curvilinear response between N loading rate (ranging between 300 and 1000 kg N ha^{-1}) and cumulative N_2O emissions. Similarly, Di and Cameron (2007) reported that increased NO_3^- leaching tended to be associated with increasing urinary N loading rates. Despite these insights, the relationship between urine N concentration, volume and the resulting N loading rates generally remain poorly-characterised and many questions remain inadequately answered: e.g. are smaller volume urination events usually more concentrated in N compared with larger event volumes, and do urine volume and N concentration interact systematically to produce a range of N loading rates with variable effect on N_2O emissions?

The chemical composition of different sheep urine events may also lead to differences in N cycling and losses at the individual patch scale (López-Aizpún et al., 2020). Urine N originates in the rumen from an imbalance between degradation of dietary N substrates and uptake of N by the rumen microbiome, leading to an excess of ammoniacal N (Gardiner et al., 2016). As a means of detoxifying systemic NH_3, urea is formed in the liver (da Silva Cardoso et al., 2019) and this comprises the main N-containing excretal product in urine (ranging from 52-94% of total-N in Dijkstra et al. 2013 and between 60-100% in Chadwick et al. 2018). Other urine constituents include hippuric acid, benzoic acid, creatine, creatinine, purine derivatives and amino acids (all N-containing except benzoic acid) (Bristow et al., 1992). Hippuric and benzoic acids have both been investigated as natural inhibitors of N_2O emissions in soil. Reductions in N_2O emissions have been reported under laboratory conditions when manipulating synthetic or real urine to increase hippuric and benzoic acid concentrations (Kool et al., 2006; van Groenigen et al., 2006; Bertram et al., 2009), although the results have not been repeated under field conditions (Clough et al., 2009; Krol et al., 2015; Ciganda et al., 2018). Varying the concentration of other non-urea nitrogen constituents has generally not been found to have an

effect on N_2O emission factors (Gardiner et al., 2018). However, da Silva Cardoso et al. (2017) found that increasing concentrations of KCl in urine produced a curvilinear response in N_2O-N emission factors, with lower emission factors at higher KCl concentrations. The authors suggest an inhibitory effect of KCl on nitrification was responsible for reduced N_2O emissions, but it could also be a non-specific salt effect. The presence of hippuric acid alongside urea was found to increase NH_3 volatilisation from urine patches compared to urea alone (Whitehead et al., 1989). Doak (1952) found that allantoin and heteroauxin in urine stimulated nitrification rates in laboratory soil. The excretion of plant secondary metabolites in urine is another mechanism by which urine composition may alter urine patch N cycling (Gardiner et al., 2017; De Klein et al., 2020; Yao et al., 2018), although how the urine metabolome varies as a function of pasture quality or season is not yet well established. In this study, untargeted primary metabolism analysis is used to assess differences in the urinary metabolomic profile.

Here, we i) assess the frequency and volume of urine events from ewes in urine collection pens, ii) investigate the interaction between urine-N concentration, urine volume and soil N loading rate; and iii) determine the site (i.e. contrasting forage quality) and seasonal differences in sheep urine chemical constituents and metabolomic profile. In addition, we use the urine composition dataset to produce an artificial sheep urine "recipe" to allow development of a standardized urine for future research. We focus on sheep as they are the main grazing animal within the study area and due to the limited data currently available for sheep. Increasing the available data on urine patch parameters will better inform process-based N cycling and greenhouse gas emission models, allowing the spatially heterogeneous return of nutrients in paddocks and their associated losses to be more accurately quantified (Hoogendoorn et al., 2010).

2. Materials and Methods

To investigate variations in sheep urine volume, frequency and chemical composition, two study sites were used at the Henfaes Research Station, Abergwyngregyn, North Wales (53°13'N, 4°0'W). The first site was a semi-improved upland (270 m a.s.l.) grassland, comprising of a mosaic of grassland vegetation classified under the British National Vegetation Classification (NVC) scheme as U4 (*Festuca ovina - Agrostris capillaris - Galium saxatile* grassland) and M56 (*Lolium perenne - Cynosurus cristatus* grassland) (Rodwell, 2000). Seasonal changes in urine parameters were investigated at this site by conducting urine collection studies over the spring, summer and autumn of 2016, which were part of a larger research project exploring urine N_2O emissions from upland pastures (Marsden et al., 2018). The second study site was a lowland (< 100 m a.s.l.) improved *Lolium multiflorum* pasture, where a urine collection study was run in the autumn of 2016, allowing a comparison of the two contrasting pastures for the autumn sampling period. A meteorological station was installed at the experimental site (Skye Instruments Ltd., Llandrindod Wells, UK), recording weather data (incoming solar radiation flux density, ambient air temperature and daily rainfall) at half-hourly intervals.

2.1 Urination event data from penned sheep

Barren Welsh Mountain ewes (n = 6) were acclimatised on their respective pastures by allowing them to graze freely for five days prior to urine collection. Sheep were contained in urine collection pens (see Fig. 1), approved by Bangor University's School of Natural Sciences Ethics Committee (Ethics approval code CNS2016DC01). The pens consisted of discrete stalls for the six sheep, in which metal hurdles separated the individual animals. Slatted flooring (Rimco Ltd., Yorkshire, UK) raised 10 cm above ground level was used to facilitate urine collection using plastic trays placed underneath the floor. A mesh screen lined with muslin was

158 placed between the collection trays and the slatted flooring to prevent faecal or other
159 contaminants (e.g. refused feed or wool) from entering the urine collection trays. The flooring
160 was regularly cleaned to remove faeces and prevent contamination of collected urine samples.
161 Water and feed buckets were also provided, with cut forage supplied to the animals during their
162 time in the pens. We did not observe the sheep drinking from the provided water during their
163 time in the apparatus. When not in the urine collection pens, the sheep were enclosed in a larger
164 grazing pen on the same pasture, which was moved around to ensure ample forage was
165 available. Quantities of feed consumption were not measured, but sheep were allowed to feed
166 *ad libitum* through the provision of forage as stated above.

167 Urine samples were collected over a period of approximately two weeks per study period, with
168 animals typically in the pens between the hours of 10:00 and 16:00. At the upland semi-
169 improved site, urine from a total of 56 individual urination events were collected from the pens
170 in the spring (over six total collection days); 40 events in the summer (over six collection days);
171 and 43 events in the autumn (over seven collection days). At the improved site, urine from 54
172 individual urination events were collected from the pens in the autumn (over four collection
173 days). Urine from entire individual urine events were collected and the volume and time of day
174 of each event recorded. Volumes were corrected for the liquid absorbed in the muslin or
175 adhered to the urine collection apparatus by applying a correction factor. This was calculated
176 by pouring known amounts of water (ranging from the smallest to largest recorded urine event
177 volumes) through the collection apparatus and calculating the recoveries (See Supplementary
178 Information 1, Fig. S1). Daily urination frequency rates were estimated by dividing the number
179 of urine events collected by the time (hours) spent in the urine collection apparatus, and
180 multiplying by 24 (assuming similar rates of urination frequency in the night periods). Urine
181 samples were stored in acid-washed polypropylene bottles in a refrigerated box immediately
182 after collection, and before handling and freezing on return to the laboratory.

183 *2.2 Analysis of urine chemical constituents*

184 In the laboratory, individual urine samples were filtered on ice through Whatman. No.1 filter
185 papers (11 µm pore size) prior to freezing to remove any large particulate matter. Subsamples
186 of each event were taken and stored frozen at -20 °C before further analysis of chemical
187 constituents. The pH and electrical conductivity (EC) of samples were measured using standard
188 electrodes. The total N and dissolved organic carbon (C) in the urine samples were measured
189 on a Multi N/C 2100S analyser (AnalytikJena AG, Jena, Germany). Urea concentrations were
190 measured via the enzymatic method of Orsenneau et al. (1992). Concentrations of NH_4^+ and
191 NO_3^- were determined colorimetrically via the methods of Mulvaney (1996) and Miranda et al.
192 (2001), respectively. Free amino acids were determined fluorometrically via the method of
193 Jones et al. (2002). Allantoin, creatinine, uric acid, hippuric acid and benzoic acid were
194 determined using a Varian Pro Star 310 HPLC System (Varian Inc., Palo Alto, CA) using a
195 C18 HyperClone® 5 µm 12 nm ODS column (250 × 4.6 mm) column (Phenomenex Inc.,
196 Cheshire, UK). Briefly, the variable wavelength detection was set at 218 nm, with a flow rate
197 of 1 mL min^{-1}, pumping mobile phase A (KH_2PO_4; 17 g L^{-1}; adjusted to pH 4) or mobile phase
198 B (60% mobile phase A and 40% HPLC-grade methanol). Urine samples were diluted in mobile
199 phase A as necessary, prior to analysis. Levels of K^+, Na^+ and Ca^{2+} were determined in the
200 urine samples using a Sherwood Model 410 flame photometer (Sherwood Scientific Ltd.,
201 Cambridge, UK).

202 *2.3 Estimation of individual urine patch N loading rates*

203 Hypothetical individual urine patch N loading rates were calculated for the collected urine
204 events. Here, in addition to the N concentration and volume of each individual urine event,
205 values for the urine-to-soil surface area influenced were required. For the semi-improved site
206 we used a ratio of 17.5 L urine m^{-2}, determined by application of Brilliant Blue dye at a typical

urine volume and measuring the wetted area by overlaying a sheet of acetate and tracing the extent of the dye across the pasture surface (see Marsden et al., 2018). The same methodology was repeated in this study for the improved site, to produce a site-specific urine-to-soil surface area ratio, where a lower ratio of 8.9 L urine m^{-2} was recorded.

2.4 Sheep urine metabolomic profile

The metabolomic profiles of urine samples were determined by syringe filtering (< 0.2 μm) and flash freezing individual urine samples from sheep in the spring (n = 5), summer (n = 5) and autumn (n = 4) on the semi-improved site and in the autumn (n = 4) on the improved field site. Procedural blanks of ultra-pure water (18.2 MΩ resistance) were syringe filtered as above and included in the analysis. Frozen samples were stored at -80 °C before being shipped on dry ice to the West Coast Metabolomics Center at UC Davis for untargeted primary metabolism analysis. Samples were analysed via ALEX-CIS GC-TOF-MS (Gerstel Inc., Linthicum, MD), see Supplementary Information 2 for details of instrument settings.

2.5 Forage analysis

Samples of the forage (n = 4) available to the sheep in each season and at each site were taken and analysed for total C and N content on a TruSpec® Analyzer (Leco Corp., St. Joseph, MI). Samples were sent to Sciantec Analytical (Cawood Scientific Ltd., North Yorkshire, UK) for nutritional analysis, including crude protein content, neutral detergent fibre (NDF), sugar, ash, metabolizable energy (ME), D value (digestible organic matter), acid detergent fibre (ADF), oil by acid hydrolysis (OAH) and neutral cellulase gammanase digestibility (NCGD).

2.6 Artificial sheep urine recipe

We updated the artificial sheep urine recipe of Lucas and Jones (2006), which was based on sheep urine data from Bathurst (1952), Bristow et al. (1992) and Anger et al. (2003). We based values on the mean concentration of the compounds measured in this study across all measured

urine events (all season and sites) and provide the total N content of each artificial urine recipe. Unmeasured compounds were kept the same as that in Lucas and Jones (2006).

2.7 Statistical analysis

Seasonal differences in the semi-improved forage analyses and urine chemical composition were assessed via ANOVA and Tukey's HSD test in R (R Core Team, 2018). Test assumptions were evaluated prior to analysis: homogeneity of variance was assessed using Levene's test ('car' package in R; Fox and Weisberg, 2011) and normality was assessed via the Shapiro-Wilk test. If assumptions were violated, a Games-Howell Test (Peters, 2018) was used in place of Tukey's HSD test. For the N loading rates a Kruskal-Wallis test was used due to violations of the equivalent parametric test. Comparisons between the improved and semi-improved forage analyses and urine composition data in autumn were compared via t-tests (after checking test assumptions).

Rates of urination frequency, volume and N excretion from the penned animals were calculated and expressed per sheep on a daily basis. Here, data were filtered to remove days where no urine was collected and two replicate sheep were removed from the analysis due to their relative infrequency of urination events (these data were assumed atypical) to avoid skewing the data set.

Metabolomics data were analysed via MetaboAnalyst v4.0 (Xia and Wishart, 2016; Chong et al., 2018) to produce heat-maps of identified and unidentified compounds. Data were $\log_{10}$-transformed prior to analysis and no missing value estimations or feature filtering were applied. Since the samples were sent in two separate batches for analysis, comparisons (t-tests) were made between spring and summer urine samples from the semi-improved pasture and between the semi-improved and improved pasture urine samples in autumn. Metabolic pathway maps

were produced in KEGG Mapper v4.0 (Kanehisa et al., 2012), where *Ovis aries* was selected as a model organism when investigating the metabolic pathways.

3. Results & Discussion

3.1 Forage analysis and influence on urine N excretion by site and season

Results for the forage analyses, displayed in Table 1, show the foliar N and crude protein content were significantly higher (t-test, $n = 8$, $p < 0.05$) in the improved pasture in the autumn compared to the semi-improved pasture in autumn. Notably, this resulted in significantly higher (t-test, $n = 89$, $p < 0.05$) estimates of daily urine N excretion between the two contrasting diets (Table 2). Total N concentration within ruminant urination events is often positively correlated with crude protein intake (Decandia et al., 2011; Dijkstra et al., 2013). Here we observed the surplus N being excreted within the urine, resulting for the same season in higher estimated overall N excretion on the higher quality forage (26.65 ± 2.32 g N $sheep^{-1}$ d^{-1}) compared to the lower quality forage (16.66 ± 2.32 g N $sheep^{-1}$ d^{-1}). When deposited to pasture, we would, therefore, expect greater overall N losses (e.g. NH_3 volatilisation, NO_3^- leaching and N_2O emissions) from the improved compared to the semi-improved site. Results of Marsden et al. (2018) also reveal low N_2O emission factors from sheep urine deposited to the same semi-improved pasture, highlighting the importance of considering contrasting soil types in combination with site-specific livestock urine when assessing urinary N losses.

The majority of forage analysis results differed significantly (Tukey's HSD; $n = 12$, $p < 0.05$) between the spring and summer at the semi-improved site (Table 1). However, the forage analyses in autumn were similar to those in both the spring and the summer samples (Tukey's HSD; $n = 12$, $p > 0.05$). Notably, no significant difference (Tukey's HSD; $n = 12$; $p > 0.05$) were observed in the crude protein contents across seasons, yet we estimated higher total N excretion in the summer and autumn compared to spring (Tukey's HSD; $n = 89$, $p < 0.05$). The

reasons for this remain unclear, but may have been linked to different patterns of grass consumption between seasons, which were not recorded in this study. Future studies of this kind should, therefore, quantify feed and water intake in order to assess the influence of these factors on urine production.

3.2 Sheep urine frequency, individual event volume and daily volume

Across all four urine collection campaigns, the sheep urination frequency was 9.7 ± 0.7 urine events sheep^{-1} d^{-1}, ranging between 4 and 31 urine events sheep^{-1} d^{-1}, assuming that the time spent in the urine collection pen was representative of a 24 h period. Sheep urination frequencies did not differ between sites (Tukey's HSD, $n = 65$, $p > 0.05$) or seasons (t-test, $n = 47$, $p > 0.05$). The rates of urination frequency were similar to those measured by Liu and Zhou (2014) in China, who reported urination frequencies in the range of 10.8 to 11.7 events d^{-1} for sheep housed in metabolism crates. Betteridge et al. (2010) used sensor data (i.e. free roaming sheep) and reported that sheep urinated 21.2 ± 6.1 (S.D) events d^{-1}, which was much higher than the frequency observed in this study. This may have been partly because Welsh Mountain ewes are a small breed of sheep, typically 10 kg lighter than those studied in Betteridge et al. (2010). Schlecht et al. (2005) visually observed 0.64 events h^{-1} in sheep during the grazing day, corresponding to 15.3 events d^{-1} assuming the grazing day is representative of a full 24 h period. Our results for urination frequency are, therefore, consistent with the range reported by other studies.

The mean individual urine event volume across the entire dataset was 289 ± 14 mL (range 46 - 933 mL). Measured data on individual urine event volumes are scarce, but typical sheep urine volumes presented by Haynes and Williams (1993) and Doak (1952) of 150 mL, are slightly lower than the mean urine event volume as measured in this study. A significantly greater (Tukey's HSD, $n = 128$, $p < 0.05$) individual urine event volume was observed in autumn at

the semi-improved site, compared to either spring or summer (Table 2). Individual urine event volumes did not differ between the semi-improved and improved pasture in the autumn (t-test; $n = 93$, $p > 0.05$). Differences in urine volume would be intuitively linked to gross water consumption upon drinking and within the forage. They may, therefore, have been influenced by contrasting temperatures (e.g. higher temperatures linked to dehydration or stimulating animals to drink more frequently) or rainfall (amount of moisture in and adhered to the pasture) in each campaign. Weather data (Supplementary Information 1, Fig. S2) revealed a slightly higher daily mean temperature in autumn at the semi-improved site (16.4 °C) compared to spring or summer (11.3 and 14.5 °C, respectively). However, the mean air temperature at the improved site in autumn was 11.4 °C. Cumulative rainfall at the semi-improved site was 7.3, 7.5, and 23.8 mm in the spring, summer and autumn urine collection periods respectively and 0.2 mm at the improved site in the autumn urine collection period. The low rainfall values are indicative of a short experimental duration and collection over dry periods. Our highest values for urine volume were recorded on the warmest and wettest days (autumn; semi-improved site) and the colder and driest days (autumn; improved site). Therefore, there does not appear to be a clear link to temperature or rainfall with urine volume in this study and we suggest monitoring water intake in future studies.

We estimated the mean of the total daily urine volume excreted across all the urine collection studies as 2.77 ± 0.15 L sheep^{-1} d^{-1} (range 0.51 - 6.84 L sheep^{-1} d^{-1}), with the same statistical trends as observed for the individual urine event volume (Table 2). Daily volume ranges reported from other studies employing metabolism crates include 0.5-3 L sheep^{-1} d^{-1} (Ledgard et al., 2008); 2.9 - 4.6 L urine sheep^{-1} d^{-1} (O'Connell et al., 2016) and an average of 2.9 L urine sheep^{-1} d^{-1} (Doak, 1952). Our values agree well with the total daily volume of urine produced per sheep per day in the cited studies. As our data for sheep urine frequencies and volumes only pertain to a ca. 6 h window of the grazing day, we suggest caution in interpretation of the 24 h

extrapolation. The fact that the sheep were stationary in the pen may have influenced these parameters. Further work on the same site has been conducted with sensor-based technology, allowing the animals to roam and graze naturally. This will help to understand whether urine frequency and volume is affected by penning for a shorter period of the day.

3.3 Interaction of urine N concentration, volume and N loading rate

The interaction between urine volume, N concentration and N loading rate for each urine collection study can be seen in Fig. 2. The mean individual urine N concentration for all treatments was 5.7 ± 0.2 g N L^{-1}, ranging between 1.2 and 13.0 g N L^{-1}. We found no correlation between the urine N concentration and urine volume, but generally urine samples tended not to have simultaneously high volume and N content (note absence of data points in top right corner of the figures). For particular lower urine volumes, there were wide ranges of N concentrations. Seasonal differences in the urine N loading rates were found (Kruskal-Wallis test, $n = 139$; $p < 0.05$). The mean calculated urine patch N loading rates in the semi-improved pasture were significantly lower in spring (794 ± 66 kg N ha^{-1}) compared to summer (1057 ± 73 kg N ha^{-1}), and autumn (966 ± 63 kg N ha^{-1}). At the improved site, the mean urine N loading rates were significantly lower (t-test; $n = 97$; $p < 0.05$) (621 ± 22 kg N ha^{-1}) compared to the semi-improved site in autumn (966 ± 63 kg N ha^{-1}), despite higher individual urine N concentrations.

Published data on the interaction between sheep urine N concentration, volume and the resulting area-specific urine patch N loading rates are scarce. Additionally, direct measurements of the urine patch wetted area are often neglected when conducting urine patch studies. Instead, the data from Haynes and Williams (1993) are often utilised for sheep (i.e. 150 ml urine to 300 cm^2 wetted area, or 5 L m^{-2}). Our tracing data with Brilliant Blue dye highlights that the urine patch wetted area can differ greatly between contrasting soil and vegetation types (17.5 L urine m^{-2} at the semi-improved site and 8.9 L urine m^{-2} at the improved site). This may

have been linked to differences in urine infiltration rates as a result of contrasting soil structure between the two sites, or contrasting vegetation (i.e. more bryophytes at the semi-improved site) resulting in a smaller wetted area at the semi-improved site. These differences resulted in lower N loading rates (smaller bubble sizes in Fig. 2) at the improved site compared to the semi-improved site, despite the higher dietary and urinary N concentrations at the improved site. Haynes and Williams (1993) report N loading rates to be in the region of 1000 kg N ha^{-1} for dairy cattle urine patches and 500 kg N ha^{-1} for sheep. Our data clearly show that a very large range in N loading rates exists for sheep urine patches (between 203 and 2283 kg N ha^{-1}). The mean urine patch N loading rate across all trials was 838 ± 31 kg N ha^{-1}, which is higher than that reported by Haynes and Williams (1993). This suggests that the N loading rates and subsequent estimates of NH_3 volatilisation, N_2O emissions and NO_3^- leaching may be underestimated from sheep in previous studies.

3.4 Individual urine event chemical properties

The variation in chemical properties for individual urine samples split by season and site (i.e. contrasting forages on offer) are shown in Table 3. Briefly, we found several significant differences in urine chemical composition between seasons and sites (see Table 3 for tests and statistical groupings). This information could be useful for modelling the N cycle in grazed pasture systems, however, further work is required to understand how variations in urine chemical composition may effect subsequent soil N cycling under urine patches, and associated N losses to the atmosphere and in runoff. Given the large variations observed, we would recommend collecting site and seasonal-specific urine for use in studies assessing N losses from the urine patch. In addition, as suggested by López-Aizpún et al. (2020), providing detail on the urine chemical composition in urine-patch N loss studies would allow for a better understanding of how changes in urine chemical composition could influence N_2O emission factors. We extend this recommendation to other losses, therefore to improve understanding of

N cycling under urine patches, detailed information on location and urine chemistry is essential. Our study could be improved by looking at urine composition in winter, where N loss risk could be higher e.g. increased rainfall resulting in greater leaching losses. In addition, providing the animals with cut forage may have reduced the opportunity for the grazing animals to roam and select forage. This may have been more of a problem at the semi-improved site, as the diversity in the vegetation was greater compared to the monoculture in the improved site. Our study sought to seek a balance between grazing naturally and time spent in the urine collection facility to minimise this potential bias.

The data for selected N-containing constituents is expressed graphically as a proportion of the total N content of the urine samples in Fig. 3. Our range of reported individual urine N contents (1.2 to 13 g N L^{-1}) are fairly consistent with other data reported in the literature. For example, Bristow et al. (1992) observed urine N contents between 3 and 13.7 g N L^{-1} in sheep fed a ryegrass/white clover pasture; Hoogendoorn et al. (2010) reported a range of 0.5 - 16.6 g N kg^{-1} in sheep grazing a common ryegrass/cocksfoot/white clover pasture and Doak (1952) reported sheep urine N concentrations between 5.7 and 12 g N L^{-1}.

Urea was the major N-containing constituent (78-85 % of the total) in urine with the proportions of total-N generally following the trend urea > allantoin > hippuric acid > creatinine > ammonium > uric acid > amino acids > nitrate across all seasons and sites studied. Our ranges reported for urea are consistent with the ranges reported elsewhere for sheep e.g. 75-93 % by Bristow et al. (1992) and 68-85 % by Doak (1952). After urea, the purine derivative allantoin was the next biggest contributor to total N (1-27 % in all trials). Again, this is approximately consistent with data reported elsewhere for sheep and cattle urine (Bristow et al., 1992; Dijkstra et al., 2013; Chadwick et al., 2018) although the range is higher than in the cattle, sheep and goat urine samples analysed by Bristow et al. (1992) (2.2 to 11.8 % of total N). Hippuric acid, which is derived from the breakdown of phenolic compounds, comprised the next largest N

401 fraction (0.2 - 34 % of urine-N). This compares with 2.6 - 7.1 % reported by Bristow et al.
402 (1992) for sheep. Creatinine, formed via degradation of creatine and creatine-phosphate
403 (Dijkstra et al. 2013) comprised 0.1 to 7.3 % of the urine N content.

404 All other nitrogenous urine constituents analysed made up less than 1 % of the total N, on
405 average. The variations in average NH_4^+ concentrations (principally a product of urea
406 hydrolysis) may have been due to differences in sample transport time to the cold store but also
407 due to cross-reactivity by organic N during sample analysis (Herrmann et al., 2005). Free amino
408 acids were a much smaller fraction of the total N content in this study (< 1%) than the fractions
409 reported by Doak (1952) and Bathurst (1952) for sheep, which ranged between 9.3 and 15.9 %
410 of the urine-N content. The disparity in the values measured for the amino acid fraction could
411 be due to improvements in specificity of more recent methods to measure amino acids. The
412 greater ranges in urine-N constituents reported in this study compared to others reflects the
413 larger sample sizes used e.g. analysing nearly 200 individual urine events compared to e.g. five
414 individual sheep urine events in Bristow et al. (1992), one event in Bathurst (1952) and 12
415 events in Doak (1952).

416 In addition, we found a strong correlation between urine N content and urine EC (proxy for
417 ionic strength) across all seasons and for both pastures (see Supplementary Information 1, Fig.
418 S3). This suggests that EC may provide the basis for a cost-effective urine N-content sensor –
419 perhaps housed in a protective funnel suspended below the animal. Refractive index
420 (Misselbrook et al., 2016; Shepherd et al., 2016) has been used to measure urine-N content in
421 a sensor worn by grazing cattle, but this unit is probably too large for use with sheep.

422 *3.5 Artificial urine recipe*

423 Utilising the urine chemical composition data from this study we provide an artificial urine
424 'recipe', as shown in Table 4. Differences from the artificial sheep urine of Lucas and Jones

(2006) include slightly higher levels of K, Na and Ca salts and greater concentrations of hippuric acid, allantoin, creatinine and uric acid. We suggest using the updated recipe in studies where it is appropriate to use synthetic urine, because it is based on data from considerably more individual urine events than were previously available. Nevertheless, urine composition should be analysed for sheep in highly contrasting agroecosystems (e.g. drylands / tropical areas). We also suggest researchers should increase the concentration of urea within the artificial urine recipe to meet experimental N loadings required.

3.6 Urine metabolomics profile

Metabolites from a broad range of metabolic pathways were detected in the urine samples as displayed in KEGG pathway maps (Supplementary Information 1, Fig. S4 and S5). This would be expected as urine represents the end-point of many metabolic processes. Notable highlighted pathways include purine and pyrimidine metabolism, fatty acid metabolism and the TCA cycle. There were 150 identified compounds and 284 compounds classed as unknowns in the seasonal metabolite data. Hierarchical clustering heat maps of urinary metabolites between spring and summer at the semi-improved site can be seen in Supplementary Information 1 (Figs. S6 and S7). Briefly, variability was high between the urinary metabolites in spring and summer, where clustering according to season was not evident in either identified or unidentified compounds. This suggests variability in metabolite concentrations between individual sheep were greater than the variability observed between seasons at the same site.

For the sheep urine collected from autumn at the semi-improved and improved site 143 metabolites were identified and 211 were classified as unknowns. Heat maps for the sheep urinary metabolites are shown in Supplementary Information 1 (Figs. S8 and S9) for sheep grazing on semi-improved and improved pasture. This displays a clear difference in the clustering of metabolite anomalies (deviation from the average) between the two pasture types.

For 32 out of the 150 identified metabolites, there was a significant difference (t-test; n = 10; $p < 0.1$) between the spring and summer urine samples. For a further nine metabolites the differences were highly significantly different with a large fold change (see Fig. 4), indicating a large difference between the absolute value of change between two group means (i.e. before normalization). A list of all the metabolites identified as significantly different for the different seasons can be found in Supplementary Information 1 (Table S1).

Of the 143 identified metabolites in the comparison between semi-improved and improved pasture, 28 were significantly different (t-test, n = 8; $p < 0.1$) and ten were both highly significantly different with a large fold change (see Fig. 5). A list of all the metabolites identified as significantly different for the two pasture types can be found in Supplementary Information 1 (Table S2). The urine metabolome may have hitherto unknown effects on N losses, our data broadly shows distinct differences between the urine metabolome in the contrasting pastures. While we provide a synthetic urine recipe in this study, we encourage the use of real sheep urine where possible, in order to fully capture the complexity in chemical composition of the urine.

4. Conclusions

A greater total daily N excretion in urine was found for animals grazing on improved compared to semi-improved pasture, suggesting greater potential N losses from intensively managed pastures. The semi-improved site had higher urine patch N loadings, but we would expect lower N_2O emissions from these areas based on previous studies. Large volume urine samples tended to be more dilute in N, but smaller volume urine samples had a wide range of N contents. The N loading rates of individual urine patches were strongly coupled with the urine patch wetted area. This should, therefore, be measured on site prior to replicating an experimental urine patch. Site and seasonal differences were detected in the urine chemical constituents, with large

variations in the metabolite profile between contrasting pastures. It is, therefore, recommended that site- and season-specific urine should be collected for use in urine patch N loss trials.

Acknowledgements

We thank the Uplands-N_2O project team for involvement with the study as part of the wider project. Additional thanks to Danielle Hunt, Rob Brown, Emily Charlotte Cooledge and Gianmarco Sanfratello for assistance with the urine collection trials, Francesca Brailsford for discussion on metabolomics data analysis and Mark Hughes, Llinos Hughes and Wil Williams for technical assistance at the research farm. This work was funded under the UK Natural Environment Research Council (NERC), grant award (NE/M015351/1).

References

Anger, M., Hoffman, C., Kühbauch, W., 2003. Nitrous oxide emissions from artificial urine patches applied to different N-fertilized swards and estimated annual N_2O emissions for differently fertilized pastures in an upland location in Germany. Soil Use Manage. 19, 104-111.

Bathurst, N.O., 1952. The amino-acids of sheep and cow urine. J. Agr. Sci. 42, 476-478.

Bertram, J.E., Clough, T.J., Sherlock, R.R., Condron, L.M., O'Callaghan, M., Wells, N.S., Ray, J.L., 2009. Hippuric acid and benzoic acid inhibition of urine derived N_2O emissions from soil. Glob. Change Biol. 15, 2067-2077.

Betteridge, K., Costall, D., Balladur, S., Upsdell, M., Umemura, K., 2010. Urine distribution and grazing behaviour of female sheep and cattle grazing a steep New Zealand hill pasture. Anim. Prod. Sci. 50, 624-629.

Bristow, A.W., Whitehead, D.C., Cockburn, J.E., 1992. Nitrogenous constituents in the urine of cattle, sheep and goats. J. Sci. Food Agric. 59, 387-394.

Chadwick, D.R., Cardenas, L.M., Dhanoa, M.S., Donovan, N., Misselbrook, T., Williams, J.R., Thorman, R.E., McGeough, K.L., Watson, C.J., Bell, M., Anthony, S.G., Rees, R.M., 2018. The contribution of cattle urine and dung to nitrous oxide emissions: Quantification of country specific emission factors and implications for national inventories. Sci. Total Environ. 635, 607-617.

Chong, J., Soufan, O., Li, C., Caraus, I., Li, S., Bourque, G., Wishart, D.S., Xia, J., 2018. MetaboAnalyst 4.0: towards more transparent and integrative metabolomics analysis. Nuc. Acids Res. 46, W486-494.

Ciganda, V.S. López-Aizpún, M., Repullo, M.A., Wu, D., Terra, J.A., Elustondo, D., Clough, T., Cardenas, L.M., 2018. Soil nitrous oxide emissions from grassland: potential inhibitor effect of hippuric acid. J. Plant Nutr. Soil Sc. 182, 40-47.

Clough, T.J., Ray, J.L., Buckthought, L.E., Calder, J., Baird, D., O'Callaghan, M., Sherlock, R.R., Condron, L.M., 2009. The mitigation potential of hippuric acid on N_2O emissions from urine patches: An in situ determination of its effect. Soil Biol. Biochem. 41, 2222-2229.

Clough, T.J., Sherlock, R.R., Mautner, M.N., Milligan, D.B., Wilson, P.F., Freeman, C.G., McEwan, M.J., 2003. Emission of nitrogen oxides and ammonia from varying rates of applied synthetic urine and correlations with soil chemistry. Aust. J. Soil Res. 41, 421-438.

da Silva Cardoso, A., Neto, A.J., Azenha, M.V., Morgado, E.S., de Figueiredo Brito, L., Janusckiewicz, E.R., Berchielli, T.T., Reis, R.A., Ruggieri, A.C., 2019. Mineral salt intake effects on faecal-N concentration and the volume and composition of beef cattle urine. Trop. Animal Health Prod. 51, 171-177.

da Silva Cardoso, A., Quintana, B.G., Janusckiewicz, E.R., Brito, L. de F., Morgado, E. da S., Reis, R.A., Ruggieri, A.C., 2017. N_2O emissions from urine-treated tropical soil: Effect

of soil moisture and compaction, urine composition, and dung addition. Catena 157, 325-332.

De Klein, C.A.M., van der Weerden, T.J., Luo, J., Cameron, K.C., Di, H.J., 2020. A review of plant options for mitigating nitrous oxide emissions from pasture-based systems. New Zeal. J. Agr. Res. 63, 29-43.

Di, H.J., Cameron, K.C., 2007. Nitrate leaching losses and pasture yields as affected by different rates of animal urine nitrogen returns and application of a nitrification inhibitor – a lysimeter study. Nutr. Cycl. Agroecosys. 79, 281-290.

Dijkstra, J., Oenema, O., van Groenigen, J.W., Spek, J.W., van Vuuren, A.M., Bannink, A., 2013. Diet effects on urine composition of cattle and N_2O emissions. Animal 7, 292-302.

Decandia, M., Atzori, A.S., Acciaro, M., Cabiddu, A., Giovanetti, V., Molina-Alcaide, E., Carro, M.D., Ranilla, M.J., Molle, G., Cannas, A., 2011. Nutritional and animal factors affecting nitrogen excretion in sheep and goats. Cihema Option Mediteérannes. 99, 201-209.

Doak, B.W., 1952. Some chemical changes in the nitrogenous constituents of urine when voided on pasture. J. Agr. Sci. 42, 162-171.

Fenn, M.E., Poth, M.A., Aber, J.D., Baron, J.S., Bormann, B.T., Johnson, D.W., Lemly, A.D., McNulty, S.G., Ryan, D.F., Stottlemeyer, R. 1998. Nitrogen excess in north American ecosystems: predisposing factors, ecosystem responses and management strategies. Ecol. Appl. 8, 706-733.

Fox, J., Weisberg, S., 2011. An R Companion to Applied Regression. Sage, Thousand Oaks, CA.

Gardiner, C.A., Clough, T.J., Cameron, K.C., Di, H.J., Edwards, G.R., de Klein, C.A.M., 2016. Potential for forage diet manipulation in New Zealand pasture ecosystems to

546 mitigate ruminant urine derived N_2O emissions: a review. New Zeal. J. Agr. Res. 59,
547 301-317.
548 Gardiner, C.A., Clough, T.J., Cameron, K.C., Di, H.J., Edwards, G.R., de Klein, C.A.M., 2017.
549 Potential inhibition of urine patch nitrous oxide emissions by *Plantago lanceolata* and
550 its metabolite aucubin. New Zeal. J. Agr. Res. 61, 495-503.
551 Gardiner, C.A., Clough, T.J., Cameron, K.C., Di, H.J., Edwards, G.R., de Klein, C.A.M., 2018.
552 Assessing the impact of non-urea ruminant urine nitrogen compounds on urine patch
553 nitrous oxide emissions. J. Environ. Qual. 47, 812-829.
554 Goulding, K.W., Bailey, N.J., Bradbury, N.J., Hargreaves, P., Howe, M., Murphy, D.V.,
555 Poulton, P.R., Wilson, T.W., 1998. Nitrogen deposition and its contribution to nitrogen
556 cycling and associated soil processes. New Phytol. 139, 49-58.
557 Harrison-Kirk, T., Thomas, S.M., Clough, T.J., Beare, M.H., van der Weerden, T.J., Meenken,
558 E.D., 2015. Compaction influences N_2O and N_2 emissions from ^{15}N-labeled synthetic
559 urine in wet soils during successive saturation/drainage cycles. Soil Biol. Biochem. 88,
560 178-188.
561 Haynes, R.J., Williams, P.H., 1993. Nutrient cycling and soil fertility in the grazed pasture
562 ecosystem. Adv. Agron. 49, 119-199.
563 Herrmann, A., Willett, V.B., Stockdale, E.A., Jones, D.L., 2005. Interference by amino acids
564 during the determination of ^{15}N ammonium in soil. Soil Biol. Biochem. 37, 1747-1750.
565 Hoogendoorn, C.J., Betteridge, K., Costall, D.A., Ledgaard, S.F., 2010. Nitrogen concentration
566 in the urine of cattle, sheep and deer grazing a common ryegrass/cocksfoot/white clover
567 pasture. New Zeal. J. Agr. Res. 53, 235-243.
568 Jones, D.L., Owen, A.G., Farrar, J.F., 2002. Simple method to enable the high resolution
569 determination of total free amino acids in soil solutions and soil extracts. Soil Biol.
570 Biochem. 34, 1893-1902.

571 Kanehisa, M., Goto, S., Sato, Y., Furumichi, M., Tanabe, M., 2012. KEGG for integration and
572 interpretation of large-scale molecular data sets. Nucl. Acids Res. 40, D109-D114.
573 Kool, D.M., Hoffland, E., Hummelink, E.W.J., van Groenigen, J.W., 2006. Increased hippuric
574 acid content of urine can reduce soil N_2O fluxes. Soil Biol. Biochem. 38, 1021-1027.
575 Krol, D.J., Forrestal, P.J., Lanigan, G.J., Richards, K.G., 2015. In situ N_2O emissions are not
576 mitigated by hippuric and benzoic acids under denitrifying conditions. Sci.
577 Total Environ. 511, 362-368.
578 Lashof, D.A., Ahuja, D.R., 1990. Relative contributions of greenhouse gas emissions to global
579 warming. Nature 344, 529-531.
580 Ledgard, S.F., Menneer, J.C., Dexter, M.M., Kear, M.J., Lindsey, S., Peters, J.S., Pacheco, D.,
581 2008. A novel concept to reduce nitrogen losses from grazed pastures by administering
582 soil nitrogen process inhibitors to ruminant animals: a study with sheep. Agr.
583 Ecosyst. Environ. 125, 148-158.
584 Liu, H., Zhou, D., 2014. Mitigation of ammonia and nitrous oxide emissions from pasture
585 treated with urine of sheep fed diets supplemented with sodium chloride. Anim. Feed
586 Sci. Tech. 192, 39-47.
587 López-Aizpún, M., Horrock, C.A., Charteris, A.F., Marsden, K.A., Ciganda, V.S., Evans, J.R.,
588 Chadwick, D.R., Cárdenas, L.M., 2020. Meta-analysis of global livestock urine-derived
589 nitrous oxide emissions from agricultural soils. Glob. Change Biol. 26, 2002-2013.
590 Lucas, S.D., Jones, D.L., 2006. Biodegradation of estrone and 17 β-estradiol in grassland soils
591 amended with animal wastes. Soil Biol. Biochem. 38, 2803-2815.
592 Marsden, K.A., Holmberg, J.A., Jones, D.L., Chadwick, D.R., 2018. Sheep urine patch N_2O
593 emissions are lower from extensively-managed than intensively-managed grasslands.
594 Agr. Ecosyst. Environ. 265, 264-274.

595 Minson, D.J., Cowper, J.L., 1966. Diurnal variations in the excretion of faeces and urine by
596 sheep fed once daily or at hourly intervals. Br. J. Nutr. 20, 757-764.
597 Miranda, K.M., Epsey, M.G., Wink, D.A., 2001. A rapid, simple, spectrophotometric method
598 for simultaneous detection of nitrate and nitrite. Nitric Oxide 5, 62-71.
599 Misselbrook, T., Fleming, H., Camp, V., Umstatter, C., Duthie, C.A., Nicoll, L., Waterhouse,
600 T., 2016. Automated monitoring of urination events from grazing cattle. Agr. Ecosyst.
601 Environ. 230, 191-198.
602 Mulvaney, R.L., 1996. Nitrogen – inorganic forms. In: Sparks, D.L. (Eds.), Methods of Soil
603 Analysis. Part 3. Madison, WI, USA: Soil Science Society of America Inc., pp. 1123-
604 1184.
605 O'Connell, C.A., Judson, H.G., Barrell, G.K., 2016. Sustained diuretic effect of plantain when
606 ingested by sheep. Proc. New Zeal. Soc. An. 76, 14-17.
607 Orsenneau, J.L., Massoubre, C., Cabanes, M., Lustenberger, P., 1992. Simple and sensitive
608 determination of urea in serum and urine. Clin. Chem. 38, 619-623.
609 Peters, G., 2018. *Userfriendlyscience: Quantitative analysis made accessible*.
610 doi: 10.17605/osf.io/txequ, R package version 0.7.2, https://userfriendlyscience.com
611 R Core Team, 2018. R: A language and environment for statistical computing. R Foundation
612 for Statistical Computing, Vienna, Australia. https://www.R-project.org/
613 Rodwell, J.S., 2000. British Plant Communities. Cambridge University Press. ISBN
614 0521797160.
615 Schlecht, E., Hiernaux, P., Kadaouré, I., Hülsebusch, C., Mahler, F., 2005. A spatio-temporal
616 analysis of forage availability and grazing excretion behaviour of herded and free grazing
617 cattle, sheep and goats in Western Niger. Agr. Ecosys. Environ. 113, 226-242.
618 Selbie, D.R., Buckthought, L.E., Shepherd, M.A., 2015. The challenge of the urine patch for
619 managing nitrogen in grazed pasture systems. Adv. Agron. 129, 229-292.

620 Selbie, D.R., Cameron, K.C., Di, H.J., Moir, J.L., Lanigan, G.J., Richards, K.G., 2014. The
621 effect of urinary nitrogen loading rate and a nitrification inhibitor on nitrous oxide
622 emissions from a temperate grassland soil. J. Agr. Sci. 152, S159-S171.
623 Shepherd, M.A., Welten, B.G., Costall, D., Cosgrove, G.P., Pirie, M., Betteridge, K., 2016.
624 Evaluation of refractive index for measuring urinary nitrogen concentration in a sensor
625 worn by grazing female cattle. New Zeal. J. Agr. Res. 60, 23-31.
626 Van Groenigen, J.W., Palermo, V., Kool, D.M., Kuikman, P.J., 2006. Inhibition of
627 denitrification and N_2O emission by urine-derived benzoic and hippuric acid. Soil
628 Biol. Biochem. 38, 2499-2502.
629 Van Groenigen, J.W., Velthof, G.L., van der Bolt, F.J.E., Vos, A., Kuikman, P.J., 2005.
630 Seasonal variation in N_2O emissions from urine patches: effects of urine concentration,
631 soil compaction and dung. Plant Soil 273, 15-27.
632 Whitehead, D.C., Lockyer, D.R., Raistrick, N., 1989. Volatilization of ammonia from urea
633 applied to soil: Influence of hippuric acid and other constituents of livestock urine. Soil
634 Biol. Biochem. 21, 803-808.
635 Xia, J., Wishart, D.S., 2016. Using MetaboAnalyst 3.0 for comprehensive metabolomics data
636 analysis. Curr. Protoc. Bioinformatics. 55: 14.10.1-14.10.91.
637 Yao, B., Di, H.J., Cameron, K.C., Podolyan, A., Shen, J., He, J., 2018. Understanding the
638 mechanisms for the lower nitrous oxide emissions from fodder beet urine compared
639 with kale urine from dairy cows. J. Soil. Sediment. 18, 85-93.
640 Zaman, M., Nguyen, M.L., 2012. How application timings of urease and nitrification inhibitors
641 affect N losses from urine patches in pastoral system. Agr. Ecosyst. Environ. 156, 37-
642 48.

Tables

Table 1 Forage analyses (n = 4) for the semi-improved (fed to sheep in spring, summer and autumn) and improved pasture (fed to sheep in autumn). Values represent means ± SEM, small letters indicate statistical groupings ($p < 0.05$; ANOVA followed by Tukey's HSD) between seasons across the semi-improved site and large letters indicate statistical groupings between the semi-improved and improved pasture in autumn ($p < 0.05$; t-test).

Pasture properties	Spring	Summer	Autumn	Autumn
Pasture type	Semi-improved	Semi-improved	Semi-improved	Improved
Foliar N content (%)	2.98 ± 0.05 b	2.35 ± 0.21 a	2.73 ± 0.12 ab A	4.23 ± 0.20 B
Foliar C-to-N ratio	15.2 ± 0.3 a	20.0 ± 1.9 b	16.7 ± 0.7 ab B	10.7 ± 0.4 A
Crude protein (g kg^{-1} DW)	163 ± 4 a	151 ± 10 a	173 ± 6 a A	237 ± 4 B
NDF (g kg^{-1})	619 ± 2 b	579 ± 3 a	583 ± 3 a B	569 ± 2 A
Sugar (g kg^{-1})	105 ± 1 a	112 ± 1 b	106 ± 1 a A	113 ± 1 B
Ash (g kg^{-1})	76.5 ± 1.0 a	90.2 ± 3.8 b	75.2 ± 3.1 a A	95.6 ± 0.4 B
ME (MJ kg^{-1})	9.30 ± 0.04 b	8.41 ± 0.11 a	8.98 ± 0.09 b A	9.85 ± 0.05 B
NCGD	574 ± 4 b	479 ± 12 a	540 ± 9 b A	633 ± 5 B
D (%)	58.1 ± 0.3 b	52.6 ± 0.7 a	56.1 ± 0.5 b A	61.6 ±` 0.3 B
ADF (g kg^{-1})	355 ± 1 b	341 ± 1 a	342 ± 1 a B	338 ± 1 A
OAH (g kg^{-1})	31.1 ± 0.1 c	26.7 ± 0.1 a	28.3 ± 0.2 b A	29.9 ± 0.4 B

Table 2 Rates of sheep urine frequency, volume (individual event and daily) and N excretion. Values represent means ± SEM (n = 193), small letters indicate statistical groupings (ANOVA) between seasons (semi-improved site) and capital letters indicates statistical groupings (T-test) based on site.

	Spring; semi-improved	Summer; semi-improved	Autumn; semi-improved	Autumn; improved
Urination frequency (urine events sheep^{-1} d^{-1})	11.5 ± 1.6	8.4 ± 1.0	8.3 ± 0.9	10.4 ± 1.6
Individual urine event volume (ml)	177 ± 15 a	239 ± 23 a	377 ± 30 b	364 ± 32
Total urine volume (L urine sheep^{-1} d^{-1})	2.03 ± 0.17 a	2.02 ± 0.20 a	3.13 ± 0.28 b	3.73 ± 0.31
Total N excreted (g N sheep^{-1} d^{-1})	9.83 ± 0.83 a	13.80 ± 1.51 b	16.66 ± 1.18 b A	26.65 ± 2.32 B

Table 3 Seasonal and dietary variation in the chemical properties of sheep (n = 6) urine events in spring (n = 56 events), summer (n = 40 events) and autumn (n = 43 events) at the semi-improved site and autumn (n = 54 events) at the improved site. Values represent mean ± SEM, small letters indicate statistical groupings ($p < 0.05$; ANOVA and Tukey's HSD) between season at the semi-improved site and capital letters indicate statistical groupings ($p < 0.05$; T-test) between the semi-improved and improved pasture sites in autumn.

	Semi-improved upland pasture						Improved lowland pasture	
Urine parameter	Spring	Proportion of N (%)	Summer	Proportion of N (%)	Autumn	Proportion of N (%)	Autumn	Proportion of N (%)
pH	7.7 ± 0.1 a	-	8.3 ± 0.0 b	-	8.2 ± 0.0 b A	-	8.6 ± 0.0 B	-
EC (mS cm^{-1})	11.7 ± 0.7 a	-	18.6 ± 1.3 c	-	14.8 ± 0.6 b	-	14.0 ± 0.4	-
Total N (g N l^{-1})	4.5 ± 0.4 a	-	6.7 ± 0.5 b	-	5.5 ± 0.4 ab A	-	7.0 ± 0.2 B	-
Dissolved organic C (g C l^{-1})	9.0 ± 0.8 a	-	13.9 ± 1.3 b	-	8.3 ± 0.5 a	-	7.9 ± 0.3	-
Urea (g l^{-1})	7.6 ± 0.7 a	77.8 ± 1.2	10.7 ± 0.9 b	77.8 ± 4.4	10.1 ± 0.7 b A	85.0 ± 1.6	12.1 ± 0.5 B	81.3 ± 1.9
Hippuric acid (g l^{-1})	6.9 ± 0.7 b	12.0 ± 0.8	7.1 ± 1.0 b	8.7 ± 1.2	2.0 ± 0.5 a	3.6 ± 0.9	2.1 ± 0.6	2.2 ± 0.6
Allantoin (g l^{-1})	1.5 ± 0.1 a	14.1 ± 0.8	2.0 ± 0.2 b	12.3 ± 1.2	1.7 ± 0.1 ab B	11.4 ± 0.6	1.0 ± 0.1 A	5.7 ± 0.5
Creatinine (mg l^{-1})	405 ± 25 b	3.9 ± 0.2	226 ± 20 a	1.8 ± 0.2	277 ± 18 a	2.0 ± 0.1	285 ± 11	1.6 ± 0.1
Uric acid (mg l^{-1})	116 ± 8 b	0.9 ± 0.1	148 ± 14 b	0.9 ± 0.1	51 ± 6 a A	0.3 ± 0.0	120 ± 7 B	0.6 ± 0.0
Benzoic acid (mg l^{-1})	245 ± 26 a	-	467 ± 63 b	-	183 ± 13 a	-	192 ± 23	-
Amino acids (mg l^{-1})	78 ± 8 b	0.3 ± 0.0	95 ± 9 b	0.3 ± 0.0	54 ± 4 a A	0.2 ± 0.0	96 ± 7 B	0.3 ± 0.0
Ammonium (mg N l^{-1})	82 ± 11 a	2.0 ± 0.1	146 ± 6 b	2.6 ± 0.2	75 ± 2 a B	1.5 ± 0.1	29.6 ± 1.9 A	0.5 ± 0.1
Nitrate (mg N l^{-1})	0.5 ± 0.0 a	0.01 ± 0.00	0.9 ± 0.1 b	0.02 ± 0.00	0.9 ± 0.1 b	0.02 ± 0.00	0.8 ± 0.4	0.01 ± 0.00
Potassium (g K l^{-1})	3.7 ± 0.4 a	-	8.2 ± 0.8 b	-	4.7 ± 0.3 a B	-	2.5 ± 0.1 A	-
Sodium (mg Na l^{-1})	890 ± 133 b	-	315 ± 59 a	-	667 ± 71 b B	-	28 ± 7 A	-
Calcium (mg Ca l^{-1})	66 ± 5 b	-	17 ± 1 a	-	19 ± 1 a A	-	37 ± 2 B	-

Table 4 Suggested artificial urine chemical composition based on the urine chemical composition data measured in this study. Table shows artificial sheep urine composition as used in Lucas and Jones (2006) and suggested artificial urine chemical composition based on urine composition data in this study (n = 188 urine samples).

Chemical constituent	Artificial sheep urine composition as used by Lucas and Jones (2006)	Updated artificial sheep urine composition
$KHCO_3$ (g L^{-1})	6.0	6.5
KCl (g L^{-1})	3.5	4.0
Na_2SO_4 (g L^{-1})	0.4	3.0
CaCl (g L^{-1})	-	0.1
Urea (g L^{-1})	6.4	6.5
Creatine (g L^{-1})	0.85	0.85[a]
Hippuric acid (g L^{-1})	1.85	4.4
Allantoin (g L^{-1})	0.6	1.5
Glycine (g L^{-1})	0.01	0.01
Creatinine (g L^{-1})	0.015	0.3
Uric acid (g L^{-1})	0.005	0.1
Hypoxanthine (g L^{-1})	0.001	0.001[a]
Ammonium chloride (g L^{-1})	0.015	0.3
Total N content (g N L^{-1})	3.6[b]	4.4[b]

[a] Creatine and hypoxanthine were not measured in the current study
[b] Note if higher N concentrations are required for experimental purposes we recommend increasing the amount of urea as desired.

Figure Legends

Figure 1 Sheep urine collection pens, showing urine collection trays, muslin covered mesh screen, slatted flooring and feed/water containers.

Figure 2 Bubble plots displaying the interaction between individual sheep urine event volumes, N contents and estimated urine patch N loading rates (expressed as bubble size) for urine events at the semi-improved site in spring (n = 56), summer (n = 40) and autumn (n = 43) and autumn at the improved site (n = 54)

Figure 3 N-containing compounds (urea, allantoin, hippuric acid, creatinine, ammonium, uric acid and amino acids) in sheep urine samples expressed as a proportion of the total urine-N content. Panel a) is displayed on a linear y-axis scale and panel b) is the same data expressed on a log(y) scale to allow visualisation of the minor N-containing chemical constituents. Note, NO_3^--N data were omitted as values were negligible. Stacked bars represent the mean values for each season and site; legend applies to both panels.

Figure 4 Volcano plot (combination of fold change and t-test) showing differences between urine metabolites from sheep fed a semi-improved (upland) pasture diet in either spring or summer. Each point represents an identified metabolite, those coloured pink indicate significant differences (t-test; $p < 0.1$) and those annotated with a label represent metabolites possessing both a small p-value and a large fold change.

Figure 5 Volcano plot (combination of fold change and t-test) showing differences between urine metabolites from sheep fed either an improved (lowland) or semi-improved (upland) pasture diet. Each point represents an identified metabolite, those coloured pink indicate significant differences (t-test; $p < 0.1$) and those annotated with a label represent metabolites possessing both a small p-value and a large fold change.

Figure 1

Figure 2

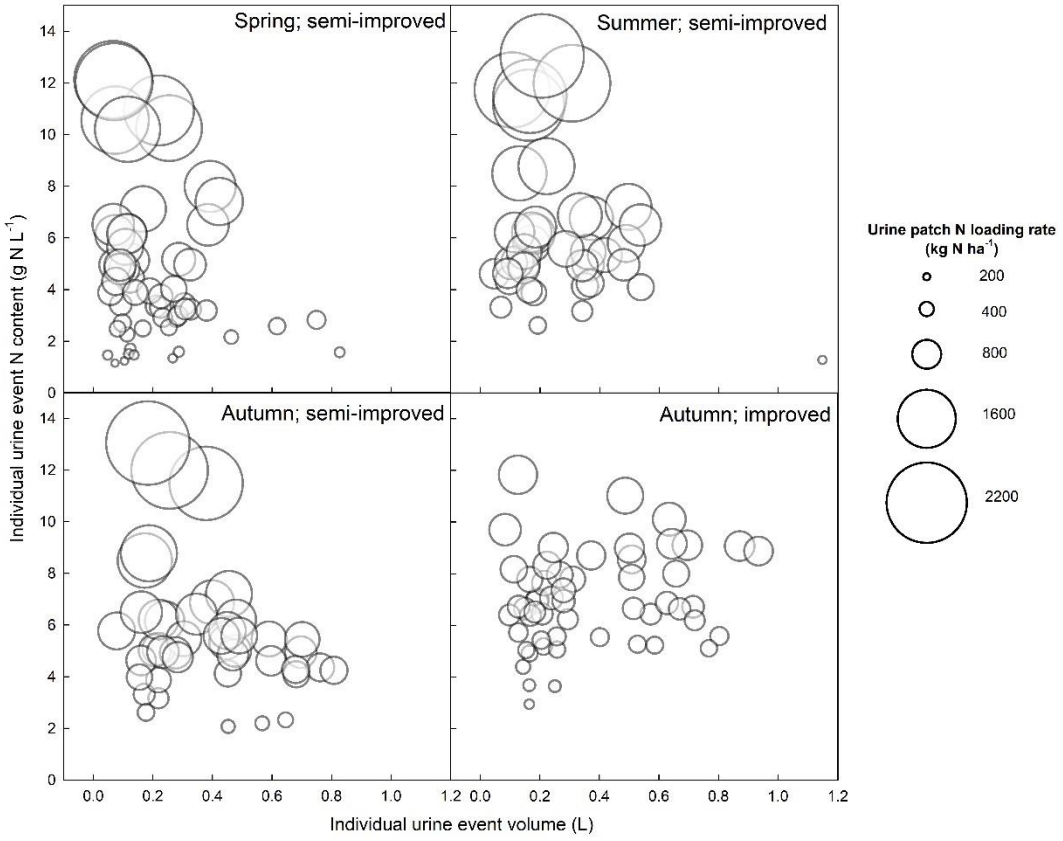

Spring; semi-improved
Summer; semi-improved
Autumn; semi-improved
Autumn; improved
Individual urine event N content (g N L-1)
Individual urine event volume (L)
Urine patch N loading rate (kg N ha-1)
200
400
800
1600
2200

Figure 3

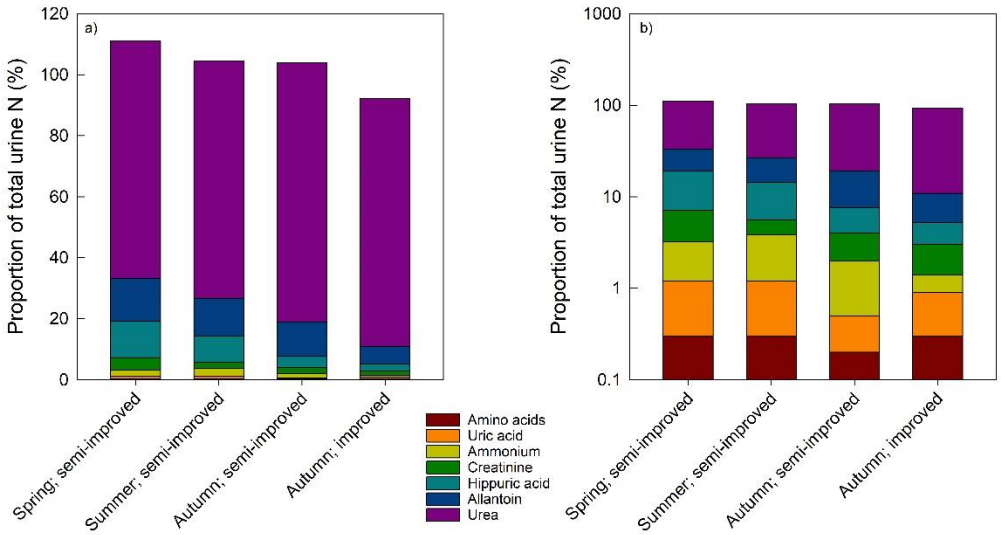


Figure 4

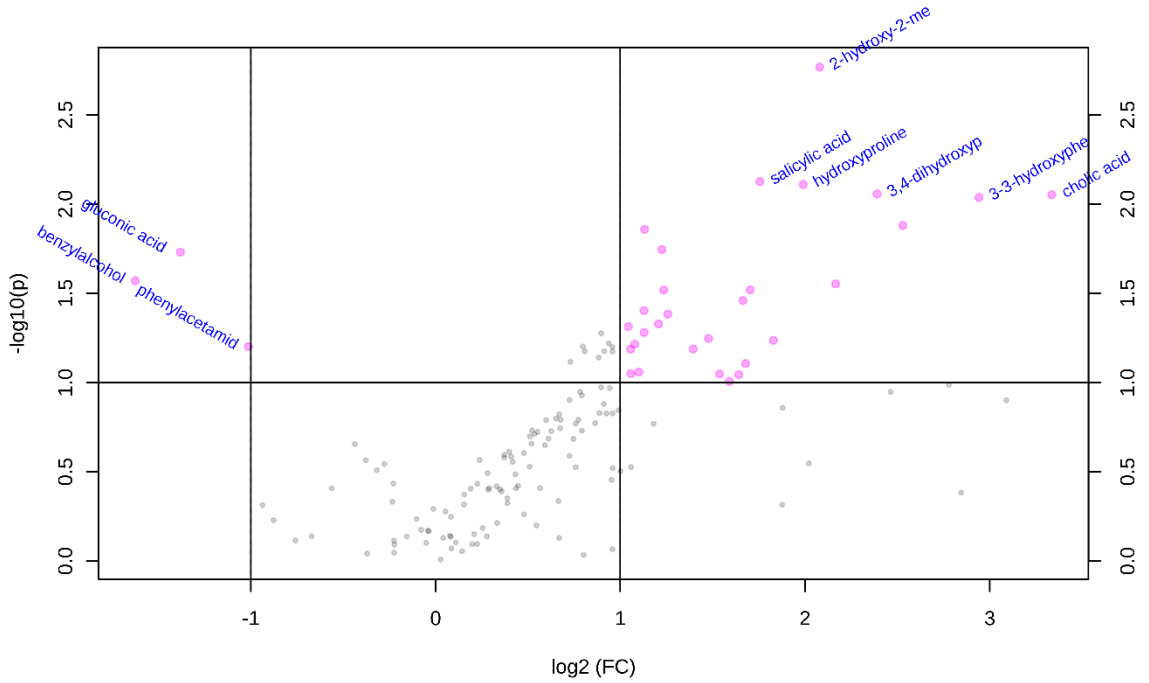
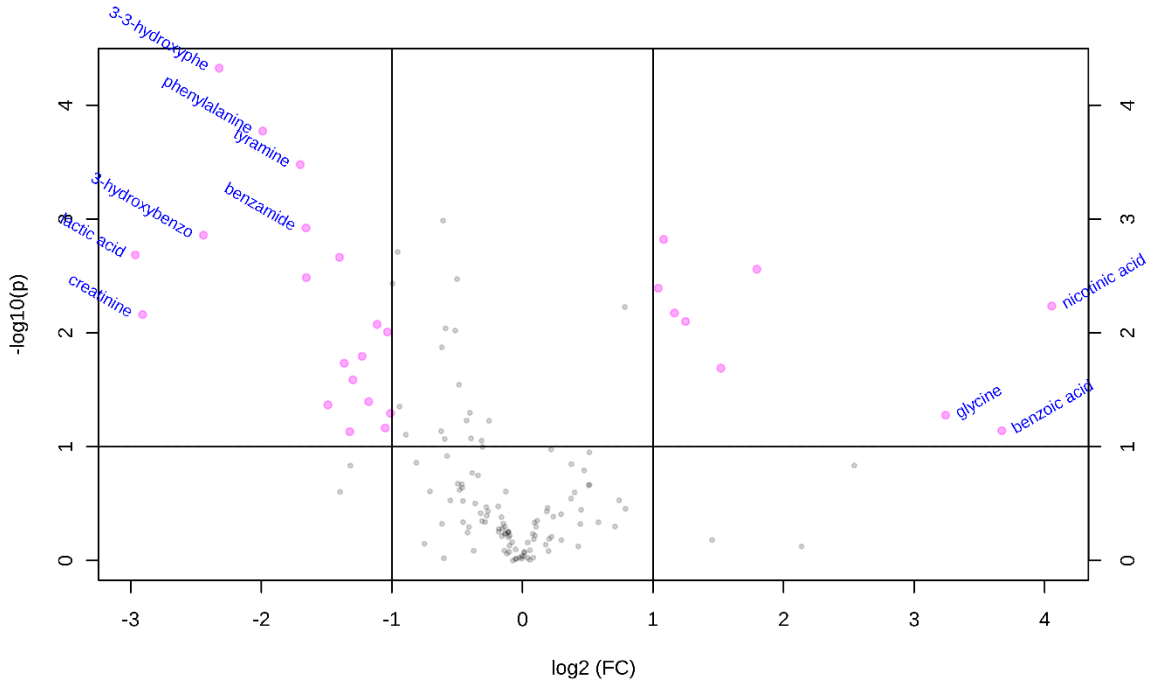


Figure 5


3-3-hydroxyphe
phenylalanine
tyramine
3-hydroxybenzo
benzamide
lactic acid
creatinine
nicotinic acid
glycine
benzoic acid
-log10(p)
log2 (FC)
0
1
2
3
4
-3
-2
-1
0
1
2
3
4

Supplementary Information 1

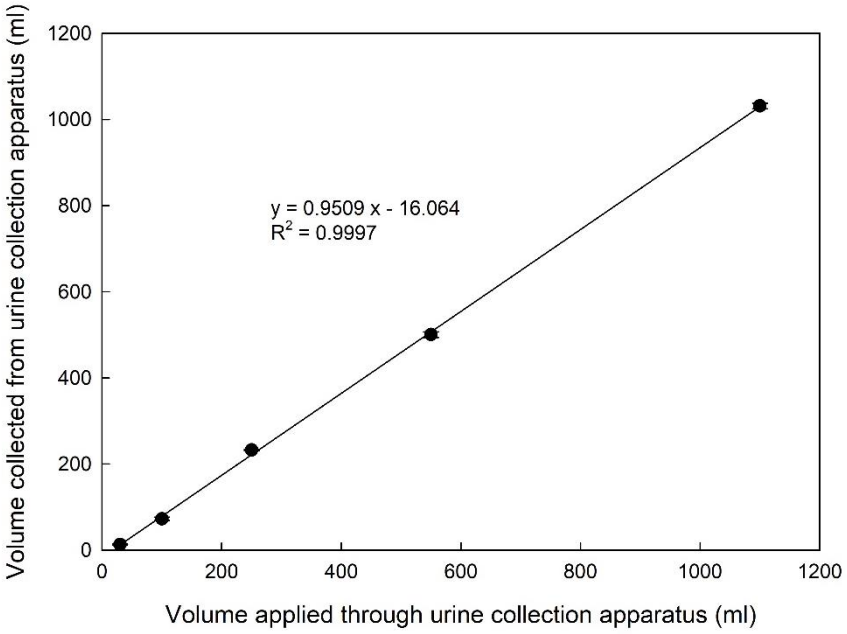


Figure S1 Recovery test of liquid poured through sheep urine collection apparatus. Symbols represent means (n = 4) and error bars denote SEM.

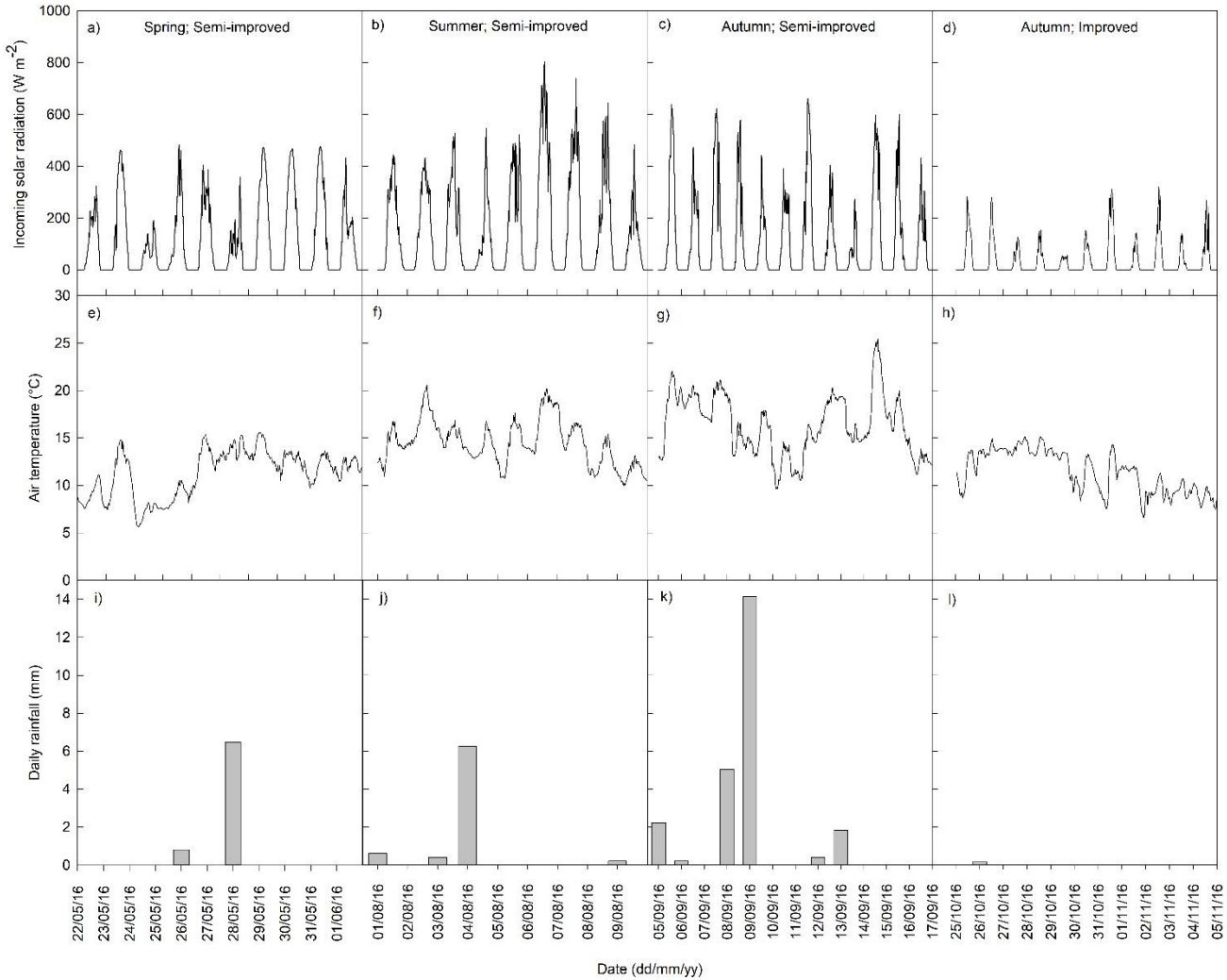


Figure S2 Weather data during the urine collection studies with penned animals including incoming solar radiation (panels a – d), air temperature (panels e – h) and daily rainfall (panels i - l). Site and season text information at the top applies to each column of panels.

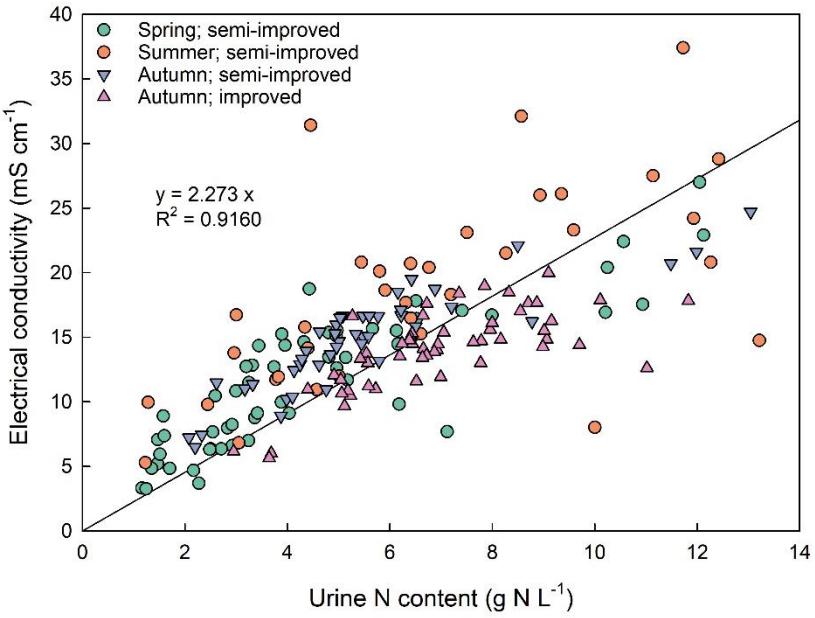


Figure S3 Correlation of urine-N content with electrical conductivity (EC) across entire urine collection dataset with penned sheep.

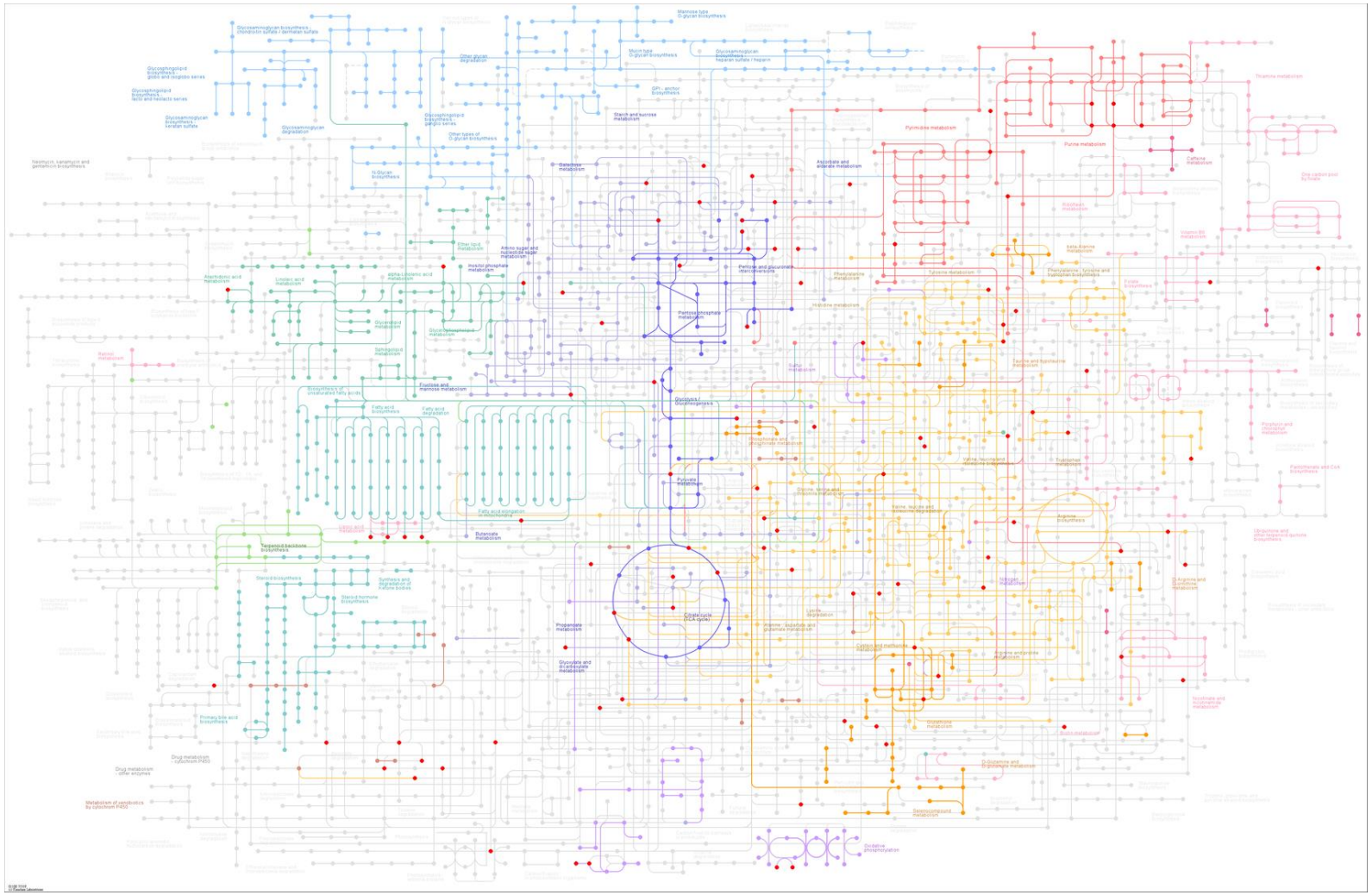


Figure S4 Metabolic pathway map highlighting (red dots) pathways detected in sheep urine samples collected in spring and summer (semi-improved pasture) using untargeted primary metabolism analysis (created using KEGG Mapper: https://www.genome.jp/kegg/mapper.html).

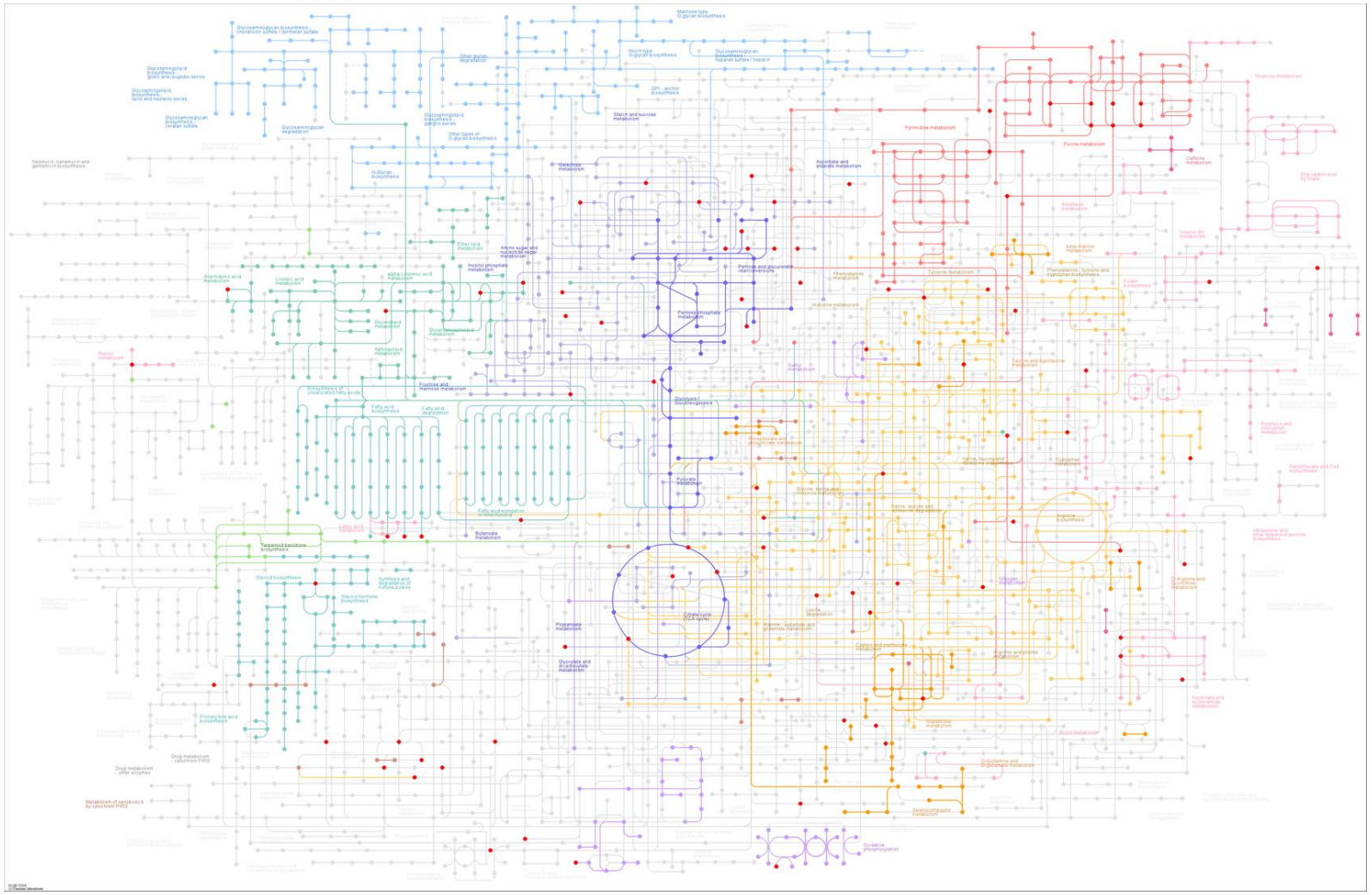


Figure S5 Metabolic pathway map highlighting (red dots) pathways detected in sheep urine samples collected from semi-improved and improved pasture diets using untargeted primary metabolism analysis (created using KEGG Mapper: https://www.genome.jp/kegg/mapper.html).

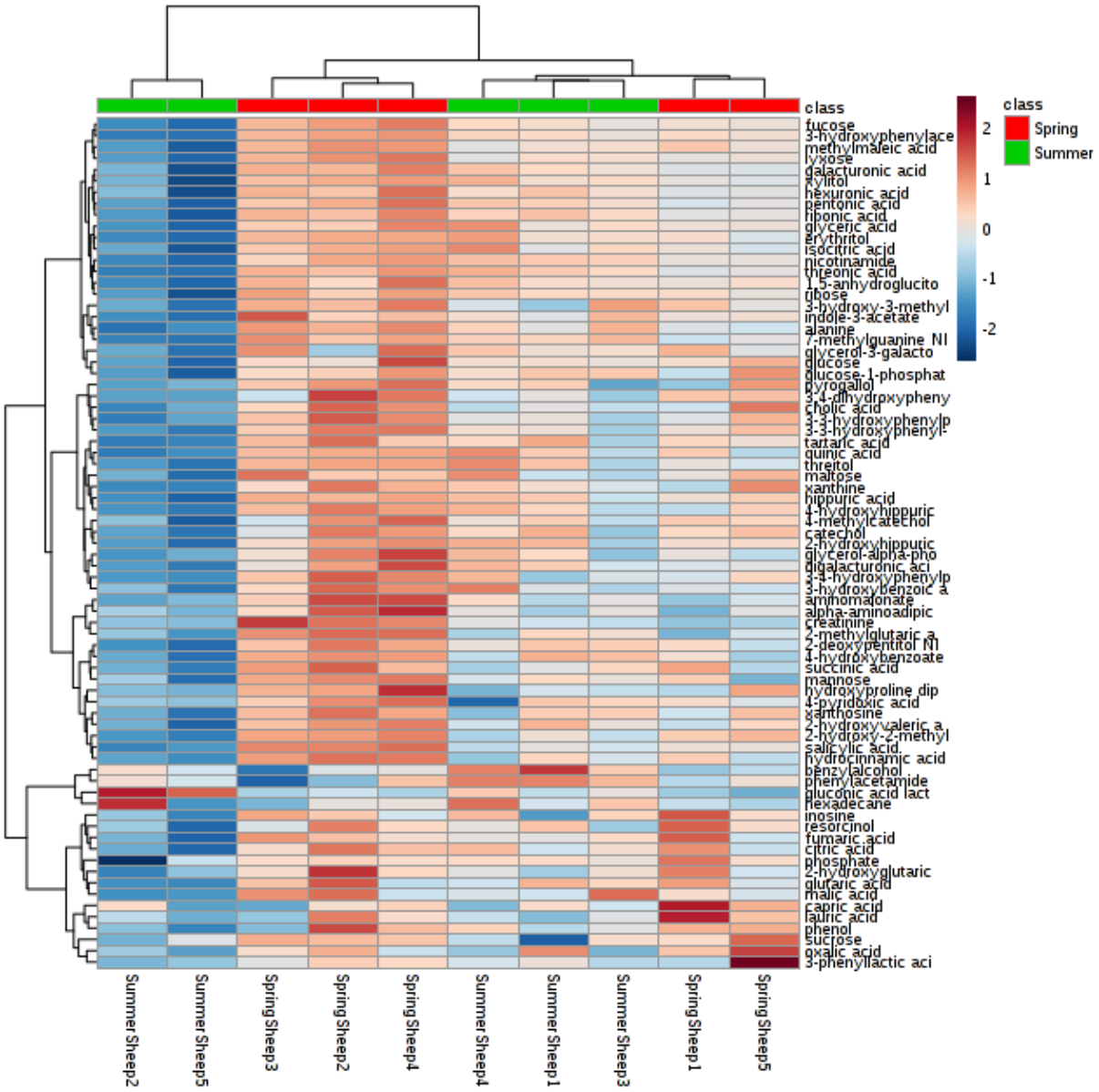


Figure S6 Heat map of mean changes in sheep urine (n = 5) primary metabolome from sheep grazing a semi-improved pasture in either spring or summer. The gradient in colour corresponds to difference in magnitude (significant decrease in metabolite displayed in blue and significant increase in metabolite showed in red) when compared with the average value. Dendrogram at the top represents clustering according to season of study and clustering of metabolites is shown by the dendrogram on the left. Metabolites are clustered by similarity according to Pearson correlation values. Only the top 75 identified metabolites (according to T-test) are displayed.

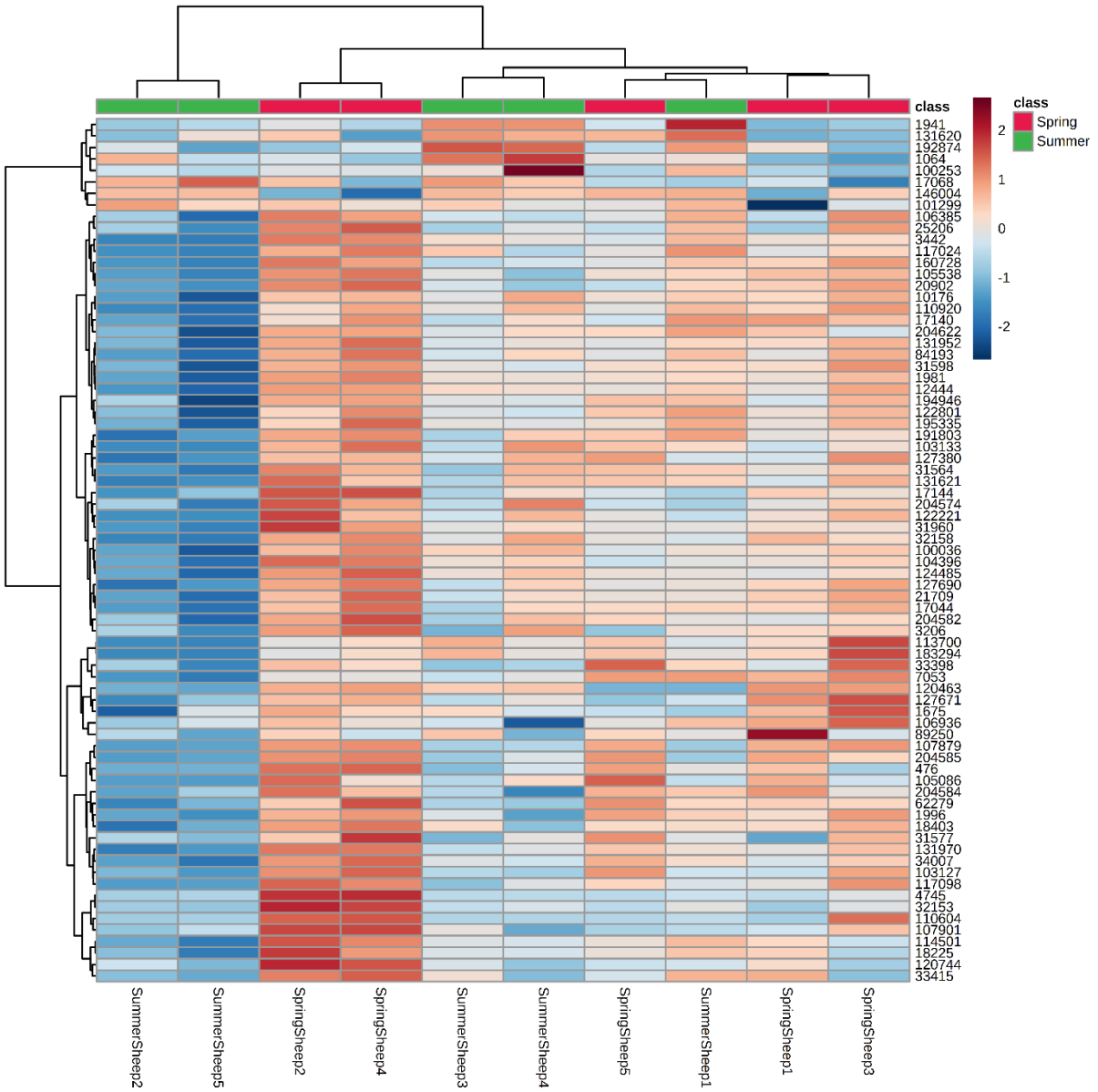


Figure S7 Heat map of mean changes in sheep urine (n = 5) primary metabolome (unidentified compounds) from sheep grazing a semi-improved pasture in either spring or summer. The gradient in colour corresponds to difference in magnitude (significant decrease in metabolite displayed in blue and significant increase in metabolite showed in red) when compared with the average value. Dendrogram at the top represents clustering according to season of study and clustering of metabolites is shown by the dendrogram on the left. Metabolites are clustered by similarity according to Pearson correlation values. Only the top 75 identified metabolites (according to T-test) are displayed.

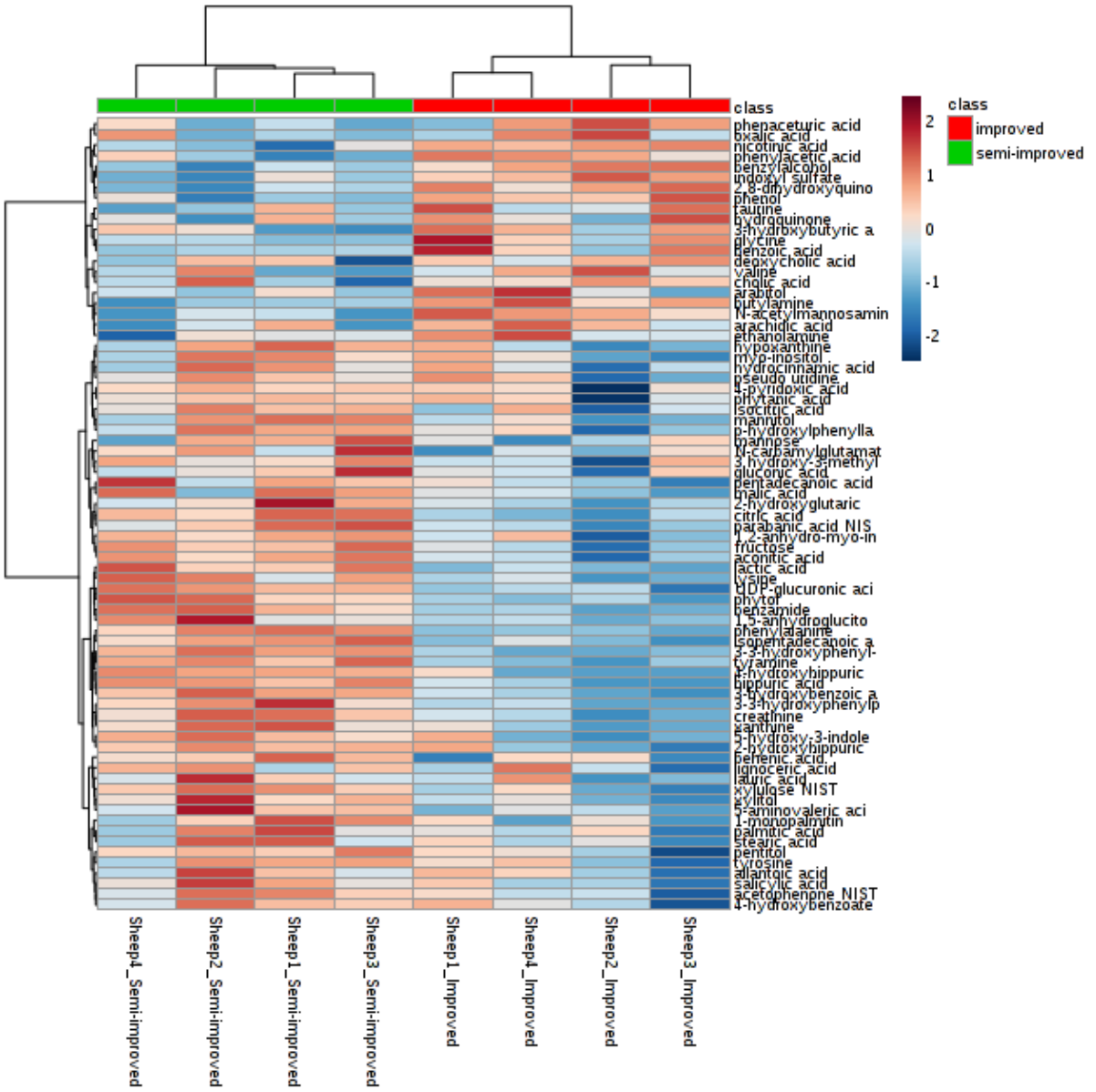


Figure S8 Heat map of mean changes in sheep urine (n = 4) primary metabolome from sheep grazing an improved (lowland) pasture or semi-improved (upland) pasture in autumn. The gradient in colour corresponds to difference in magnitude (significant decrease in metabolite displayed in blue and significant increase in metabolite showed in red) when compared with the average value. Dendrogram at the top represents clustering according to site of study and clustering of metabolites is shown by the dendrogram on the left. Metabolites are clustered by similarity according to Pearson correlation values. Only the top 75 identified metabolites (according to T-test) are displayed.

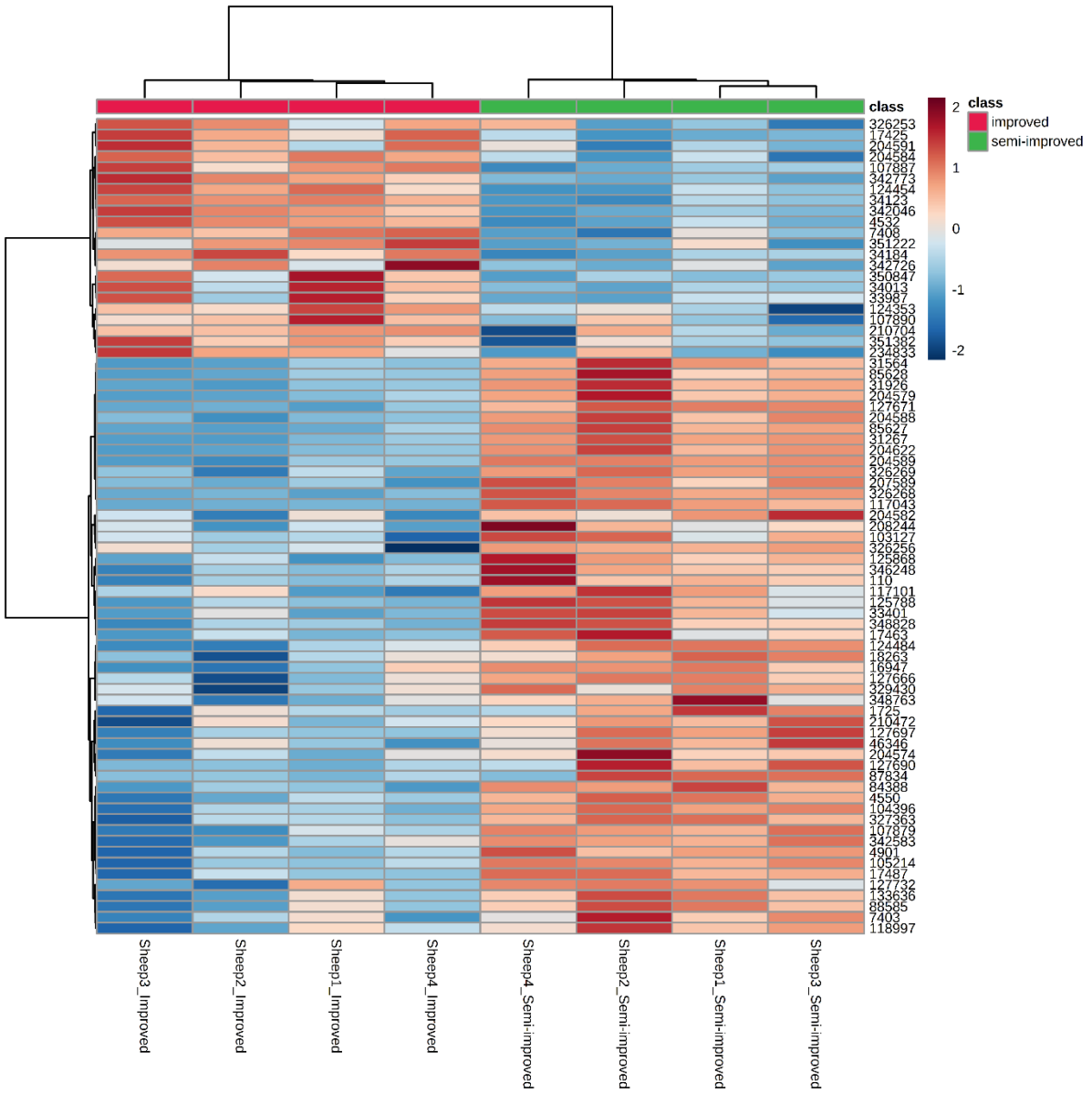


Figure S9 Heat map of mean changes in sheep urine (n = 4) primary metabolome (unidentified compounds) from sheep grazing an improved (lowland) pasture or semi-improved (upland) pasture in autumn. The gradient in colour corresponds to difference in magnitude (significant decrease in metabolite displayed in blue and significant increase in metabolite showed in red) when compared with the average value. Dendrogram at the top represents clustering according to site of study and clustering of metabolites is shown by the dendrogram on the left. Metabolites are clustered by similarity according to Pearson correlation values. Only the top 75 identified metabolites (according to T-test) are displayed.

Table S1 Metabolites identified as significantly different ($p < 0.1$; T-test) between sheep urine samples collected in spring and summer from a semi-improved pasture diet. FC stands for fold change.

Metabolite	FC	log2(FC)	p-value	$-\log_{10}(p)$
2-hydroxy-2-methylbutanoic acid	4.2251	2.079	0.001711	2.7667
salicylic acid	3.3756	1.7551	0.007487	2.1257
hydroxyproline dipeptide NIST	3.9714	1.9897	0.007763	2.1099
3,4-dihydroxyphenylacetic acid	5.2411	2.3899	0.00879	2.056
cholic acid	10.093	3.3353	0.008876	2.0518
3-3-hydroxyphenylpropionic acid	7.6815	2.9414	0.009188	2.0368
3-3-hydroxyphenyl-3-hydroxypropionic acid nist	5.7692	2.5284	0.013199	1.8795
sucrose	2.191	1.1316	0.01387	1.8579
hydrocinnamic acid	2.3381	1.2253	0.017997	1.7448
gluconic acid lactone	0.38396	-1.381	0.018639	1.7296
benzylalcohol	0.32426	-1.6248	0.026887	1.5705
succinic acid	4.4873	2.1658	0.027989	1.553
2-hydroxyglutaric acid	3.2565	1.7033	0.03031	1.5184
methylmaleic acid	2.3541	1.2352	0.03035	1.5178
3-4-hydroxyphenylpropionic acid	3.1669	1.6631	0.034757	1.459
3-hydroxy-3-methylglutaric acid	2.1856	1.128	0.039512	1.4033
lauric acid	2.389	1.2564	0.041345	1.3836
xanthosine	2.309	1.2072	0.046993	1.328
lyxose	2.0613	1.0435	0.048583	1.3135
fucose	2.1868	1.1288	0.052411	1.2806
3-hydroxyphenylacetic acid	2.7845	1.4774	0.056618	1.247
4-methylcatechol	3.5517	1.8285	0.058052	1.2362
glucose	2.1112	1.0781	0.061077	1.2141
phenylacetamide	0.49565	-1.0126	0.062876	1.2015
4-pyridoxic acid	2.6278	1.3939	0.064897	1.1878
pyrogallol	2.0788	1.0557	0.065006	1.187
xanthine	3.2012	1.6786	0.07831	1.1062
4-hydroxyhippuric acid NIST	2.1437	1.1001	0.087467	1.0582
2-hydroxyvaleric acid	2.0809	1.0572	0.089054	1.0503
citric acid	2.9025	1.5373	0.089543	1.048
indole-3-acetate	3.1203	1.6417	0.090389	1.0439
creatinine	3.0107	1.5901	0.098511	1.0065

Table S2 Metabolites identified as significantly different ($p < 0.1$; T-test) between sheep urine samples collected from either a semi-improved or improved pasture diet. FC stands for fold change.

Metabolite	FC	log2(FC)	p-value	$-\log_{10}(p)$
3-3-hydroxyphenyl-3-hydroxypropionic acid nist	0.19989	-2.3228	4.73E-05	4.3254
phenylalanine	0.2519	-1.9891	0.000169	3.7728
tyramine	0.30755	-1.7011	0.000332	3.4789
benzamide	0.31692	-1.6578	0.001198	2.9215
3-hydroxybenzoic acid	0.1839	-2.443	0.001387	2.858
butylamine	2.1164	1.0816	0.001511	2.8206
lactic acid	0.12808	-2.9649	0.002061	2.686
citric acid	0.37844	-1.4019	0.00217	2.6636
benzylalcohol	3.4702	1.795	0.002763	2.5586
phytol	0.31743	-1.6555	0.00327	2.4855
2,8-dihydroxyquinoline	2.0566	1.0403	0.004053	2.3922
nicotinic acid	16.612	4.0542	0.005813	2.2356
indoxyl sulfate	2.2416	1.1645	0.00669	2.1746
creatinine	0.13311	-2.9093	0.006913	2.1604
phenol	2.3765	1.2488	0.00794	2.1002
3-3-hydroxyphenylpropionic acid	0.46245	-1.1126	0.008428	2.0743
lysine	0.48828	-1.0342	0.00984	2.007
parabanic acid NIST	0.42686	-1.2282	0.016067	1.7941
xanthine	0.38823	-1.365	0.018461	1.7337
phenylacetic acid	2.8674	1.5197	0.020435	1.6896
1,5-anhydroglucitol	0.40655	-1.2985	0.025839	1.5877
5-hydroxy-3-indoleacetic acid	0.44206	-1.1777	0.040142	1.3964
5-aminovaleric acid	0.35589	-1.4905	0.042945	1.3671
2-hydroxyglutaric acid	0.49639	-1.0104	0.050947	1.2929
glycine	9.4514	3.2405	0.052902	1.2765
behenic acid	0.48259	-1.0511	0.068544	1.164
benzoic acid	12.744	3.6718	0.072282	1.141
N-carbamylglutamate	0.39981	-1.3226	0.073844	1.1317

Supplementary Information 2

Updated July 18, 2012. GC-TOF Operation. Metabolomics Core and Research Laboratories. UCD Genome Center, Davis, CA.

GC-TOF Method:

Instruments:

Gerstel CIS4 –with dual MPS Injector/ Agilent 6890 GC- Pegasus III TOF MS

Injector conditions:

Agilent 6890 GC is equipped with a Gerstel automatic liner exchange system (ALEX) that includes a multipurpose sample (MPS2) dual rail, and a Gerstel CIS cold injection system (Gerstel, Muehlheim, Germany) with temperature program as follows: 50°C to 275°C final temperature at a rate of 12 °C/s and hold for 3 minutes. Injection volume is 0.5 µl with 10 µl/s injection speed on a splitless injector with purge time of 25 seconds. Liner (Gerstel #011711-010-00) is changed after every 10 samples, (using the Maestro1 Gerstel software vs. 1.1.4.18). Before and after each injection, the 10 µl injection syringe is washed three times with 10 µl ethyl acetate.

Gas Chromatography conditions:

A 30 m long, 0.25 mm i.d. Rtx-5Sil MS column (0.25 µm 95% dimethyl 5% diphenyl polysiloxane film) with additional 10 m integrated guard column is used (Restek, Bellefonte PA). 99.9999% pure Helium with built-in purifier (Airgas, Radnor PA) is set at constant flow of 1 ml/min. The oven temperature is held constant at 50°C for 1 min and then ramped at 20°C/min to 330°C at which it is held constant for 5 min.

Mass spectrometer settings:

A Leco Pegasus IV time of flight mass spectrometer is controlled by the Leco ChromaTOF software vs. 2.32 (St. Joseph, MI). The transfer line temperature between gas chromatograph and mass spectrometer is set to 280°C. Electron impact ionization at 70V is employed with an

ion source temperature of 250°C. Acquisition rate is 17 spectra/second, with a scan mass range of 85-500 Da.