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



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## 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 8–11 times  $d^{-1}$ , assuming time within pens represented a 24 h period. The mean urine event volume recorded was  $289 \pm 14$  mL, from which we estimated a daily urine production value of  $2.77 \pm 0.15$  L urine sheep $^{-1} d^{-1}$ . Daily urine N excretion and individual urine N concentrations were greater from sheep in improved pasture ( $26.7 \pm 2.3$  g N sheep $^{-1} d^{-1}$ ;  $7.0 \pm 0.2$  g N L $^{-1}$ ) compared to those in semi-improved pasture ( $16.7 \pm 1.2$  g N sheep $^{-1} d^{-1}$ ;  $5.5 \pm 0.4$  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.

## 1. Introduction

The urine patches of grazing animals are well recognised hotspots of nitrogen (N) losses to the environment, including ammonia (NH<sub>3</sub>) volatilisation, nitrate (NO<sub>3</sub><sup>-</sup>) leaching and the transfer of nitrogen oxides (NO<sub>x</sub>), nitrous oxide (N<sub>2</sub>O) and nitrogen gas (N<sub>2</sub>) 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<sub>2</sub> 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-

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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<sup>-1</sup>) and cumulative N<sub>2</sub>O emissions. Similarly, Di and Cameron (2007) reported that increased NO<sub>3</sub>-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<sub>2</sub>O 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<sub>3</sub>, 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 to 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<sub>2</sub>O emissions in soil (Kool et al., 2006; Bertram et al., 2009; Krol et al., 2015). Reductions in N<sub>2</sub>O 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<sub>2</sub>O 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<sub>2</sub>O-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<sub>2</sub>O emissions, but it could also be a non-specific salt effect. The presence of hippuric acid alongside urea was found to increase NH<sub>3</sub> 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 metabolome 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* - *Agrostis capillaris* - *Galium saxatile* grassland) and MG6 (*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<sub>2</sub>O 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 placed between the collection trays and the slatted flooring to prevent faecal or other contaminants (e.g. refused feed or wool) from entering the urine collection trays. The flooring was regularly cleaned to remove faeces and prevent contamination of collected urine samples. Water and feed buckets were also provided, with cut forage supplied to the animals during their time in the pens. We did not observe the sheep drinking from the provided water during their time in the apparatus. When not in the urine collection pens, the sheep were enclosed in a larger grazing pen on the same pasture, which was moved around to ensure ample forage was available. Quantities of feed consumption were not measured, but sheep were allowed to feed *ad libitum* through the provision of forage as stated above.

Urine samples were collected over a period of approximately two weeks per study period, with animals typically in the pens between the hours of 10:00 and 16:00. At the upland semi-improved site, urine from a total of 56 individual urination events were collected from the pens in the spring (over six total collection days); 40 events in the summer (over six collection days); and 43 events in the autumn (over seven collection days). At the improved site, urine from 54 individual urination events were collected from the pens in the autumn (over four collection days). Urine from entire individual urine events were



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

collected and the volume and time of day of each event recorded. Volumes were corrected for the liquid absorbed in the muslin or adhered to the urine collection apparatus by applying a correction factor. This was calculated by pouring known amounts of water (ranging from the smallest to largest recorded urine event volumes) through the collection apparatus and calculating the recoveries (See Supplementary Information 1, Fig. S1). Daily urination frequency rates were estimated by dividing the number of urine events collected by the time (hours) spent in the urine collection apparatus, and multiplying by 24 (assuming similar rates of urination frequency in the night periods). Urine samples were stored in acid-washed polypropylene bottles in a refrigerated box immediately after collection, and before handling and freezing on return to the laboratory.

## 2.2. Analysis of urine chemical constituents

In the laboratory, individual urine samples were filtered on ice through Whatman No.1 filter papers (11  $\mu\text{m}$  pore size) prior to freezing to remove any large particulate matter. Subsamples of each event were taken and stored frozen at  $-20\text{ }^{\circ}\text{C}$  before further analysis of chemical constituents. The pH and electrical conductivity (EC) of samples were measured using standard electrodes. The total N and dissolved organic carbon (C) in the urine samples were measured on a Multi N/C 2100S analyser (AnalytikJena AG, Jena, Germany). Urea concentrations were measured via the enzymatic method of Orsenneau et al. (1992). Concentrations of  $\text{NH}_4^+$  and  $\text{NO}_3^-$  were determined colorimetrically via the methods of Mulvaney (1996) and Miranda et al. (2001), respectively. Free amino acids were determined fluorometrically via the method of Jones et al. (2002). Allantoin, creatinine, uric acid, hippuric acid and benzoic acid were determined using a Varian Pro Star 310 HPLC System (Varian Inc., Palo Alto, CA) using a C18 HyperClone<sup>®</sup> 5  $\mu\text{m}$  12 nm ODS column (250  $\times$  4.6 mm) column (Phenomenex Inc., Cheshire, UK). Briefly, the variable wavelength detection was set at 218 nm, with a flow rate of 1  $\text{mL min}^{-1}$ , pumping mobile phase A ( $\text{KH}_2\text{PO}_4$ ; 17  $\text{g L}^{-1}$ ; adjusted to pH 4) or mobile phase B (60 % mobile phase A and 40 % HPLC-grade methanol). Urine samples were diluted in mobile phase A as necessary, prior to analysis. Levels of  $\text{K}^+$ ,  $\text{Na}^+$  and  $\text{Ca}^{2+}$  were determined in the urine samples using a Sherwood Model 410 flame photometer (Sherwood Scientific Ltd., Cambridge, UK).

## 2.3. Estimation of individual urine patch N loading rates

Hypothetical individual urine patch N loading rates were calculated for the collected urine events. Here, in addition to the N concentration and volume of each individual urine event, values for the urine-to-soil surface area influenced were required. For the semi-improved site we used a ratio of 17.5  $\text{L urine m}^{-2}$ , 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  $\text{L urine m}^{-2}$  was recorded.

## 2.4. Sheep urine metabolomic profile

The metabolomic profiles of urine samples were determined by syringe filtering ( $< 0.2\text{ }\mu\text{m}$ ) 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  $\text{M}\Omega$  resistance) were syringe filtered as above and included in the analysis. Frozen samples were stored at  $-80\text{ }^{\circ}\text{C}$  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<sup>®</sup> Analyzer (Leco Corp., St. Joseph, MI). Samples were sent to Sciencetec 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 ( $26.65 \pm 2.32$  g N sheep<sup>-1</sup> d<sup>-1</sup>) compared to the lower quality forage ( $16.66 \pm 2.32$  g N sheep<sup>-1</sup> d<sup>-1</sup>). When deposited to pasture, we would, therefore, expect greater overall N losses (e.g. NH<sub>3</sub> volatilisation, NO<sub>3</sub><sup>-</sup> leaching and N<sub>2</sub>O emissions) from the improved compared to the semi-improved site. Results of Marsden et al. (2018) also reveal low N<sub>2</sub>O 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  $9.7 \pm 0.7$  urine events sheep<sup>-1</sup> d<sup>-1</sup>, ranging between 4 and 31 urine events sheep<sup>-1</sup> d<sup>-1</sup>, 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 10.8–11.7 events d<sup>-1</sup> for sheep housed in metabolism crates. Betteridge et al. (2010) used sensor data (i.e. free roaming sheep) and reported that sheep urinated  $21.2 \pm 6.1$  (S.D) events d<sup>-1</sup>, 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<sup>-1</sup> in sheep during the grazing day, corresponding to 15.3 events d<sup>-1</sup> 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  $289 \pm 14$  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

**Table 1**

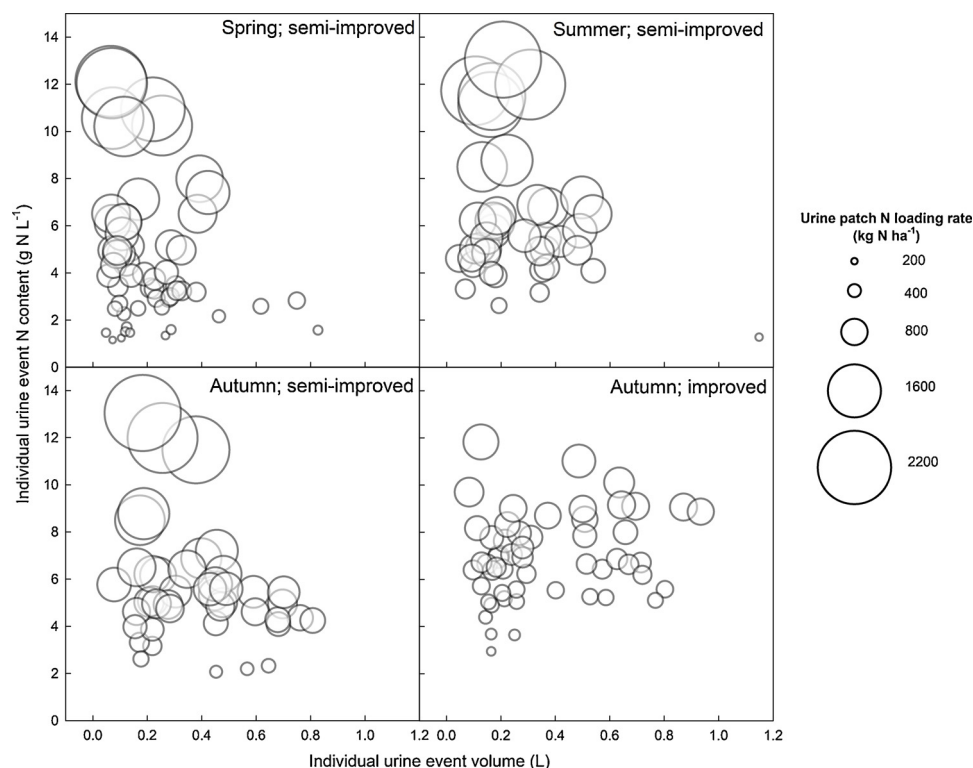
Forage analyses ( $n = 4$ ) for the semi-improved (fed to sheep in spring, summer and autumn) and improved pasture (fed to sheep in autumn). Values represent means  $\pm$  SEM, different small letters indicate a significant difference and similar letters indicate no statistical difference ( $p < 0.05$ ; ANOVA followed by Tukey's HSD) between seasons across the semi-improved site. Large different letters indicate significantly different 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 (%)	$2.98 \pm 0.05$ b	$2.35 \pm 0.21$ a	$2.73 \pm 0.12$ ab A	$4.23 \pm 0.20$ B
Foliar C-to-N ratio	$15.2 \pm 0.3$ a	$20.0 \pm 1.9$ b	$16.7 \pm 0.7$ ab B	$10.7 \pm 0.4$ A
Crude protein (g kg <sup>-1</sup> DW)	$163 \pm 4$ a	$151 \pm 10$ a	$173 \pm 6$ a A	$237 \pm 4$ B
NDF (g kg <sup>-1</sup> )	$619 \pm 2$ b	$579 \pm 3$ a	$583 \pm 3$ a B	$569 \pm 2$ A
Sugar (g kg <sup>-1</sup> )	$105 \pm 1$ a	$112 \pm 1$ b	$106 \pm 1$ a A	$113 \pm 1$ B
Ash (g kg <sup>-1</sup> )	$76.5 \pm 1.0$ a	$90.2 \pm 3.8$ b	$75.2 \pm 3.1$ a A	$95.6 \pm 0.4$ B
ME (MJ kg <sup>-1</sup> )	$9.30 \pm 0.04$ b	$8.41 \pm 0.11$ a	$8.98 \pm 0.09$ b A	$9.85 \pm 0.05$ B
NCGD	$574 \pm 4$ b	$479 \pm 12$ a	$540 \pm 9$ b A	$633 \pm 5$ B
D (%)	$58.1 \pm 0.3$ b	$52.6 \pm 0.7$ a	$56.1 \pm 0.5$ b A	$61.6 \pm 0.3$ B
ADF (g kg <sup>-1</sup> )	$355 \pm 1$ b	$341 \pm 1$ a	$342 \pm 1$ a B	$338 \pm 1$ A
OAH (g kg <sup>-1</sup> )	$31.1 \pm 0.1$ c	$26.7 \pm 0.1$ a	$28.3 \pm 0.2$ b A	$29.9 \pm 0.4$ B

**Table 2**

Rates of sheep urine frequency, volume (individual event and daily) and N excretion. Values represent means  $\pm$  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 <sup>-1</sup> d <sup>-1</sup> )	11.5 $\pm$ 1.6	8.4 $\pm$ 1.0	8.3 $\pm$ 0.9	10.4 $\pm$ 1.6
Individual urine event volume (ml)	177 $\pm$ 15 a	239 $\pm$ 23 a	377 $\pm$ 30 b	364 $\pm$ 32
Total urine volume (L urine sheep <sup>-1</sup> d <sup>-1</sup> )	2.03 $\pm$ 0.17 a	2.02 $\pm$ 0.20 a	3.13 $\pm$ 0.28 b	3.73 $\pm$ 0.31
Total N excreted (g N sheep <sup>-1</sup> d <sup>-1</sup> )	9.83 $\pm$ 0.83 a	13.80 $\pm$ 1.51 b	16.66 $\pm$ 1.18 b A	26.65 $\pm$ 2.32 B



**Fig. 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 ( $n = 54$ ).

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  $2.77 \pm 0.15$  L sheep<sup>-1</sup> d<sup>-1</sup> (range 0.51–6.84 L sheep<sup>-1</sup> d<sup>-1</sup>), 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<sup>-1</sup> d<sup>-1</sup> (Ledgard et al., 2008); 2.9–4.6 L urine sheep<sup>-1</sup> d<sup>-1</sup> (O'Connell et al., 2016) and an average of 2.9 L urine sheep<sup>-1</sup> d<sup>-1</sup> (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  $5.7 \pm 0.2$  g N L<sup>-1</sup>, ranging between 1.2 and 13.0 g N L<sup>-1</sup>. We found no

**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  $\pm$  SEM, different small letters indicate significant differences ( $p < 0.05$ ; ANOVA and Tukey's HSD) between season at the semi-improved site and different capital letters indicate statistical differences ( $p < 0.05$ ; t-test) between the semi-improved and improved pasture sites in autumn.

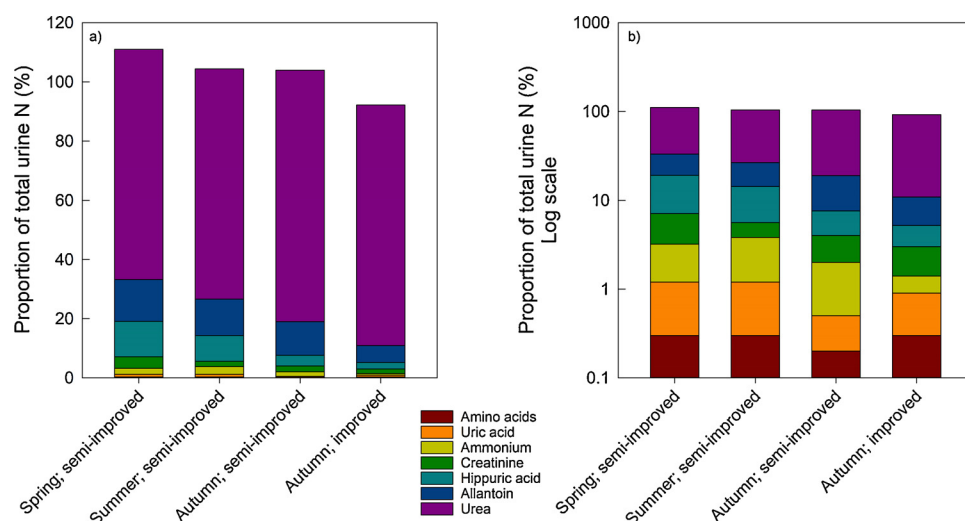
Urine parameter	Semi-improved upland pasture				Improved lowland pasture			
	Spring		Summer		Autumn		Autumn	
	Proportion of N (%)	Proportion of N (%)	Proportion of N (%)	Proportion of N (%)	Proportion of N (%)	Proportion of N (%)	Proportion of N (%)	Proportion of N (%)
pH	7.7 $\pm$ 0.1 a	-	8.3 $\pm$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

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 ( $794 \pm 66$  kg N ha<sup>-1</sup>) compared to summer ( $1057 \pm 73$  kg N ha<sup>-1</sup>), and autumn ( $966 \pm 63$  kg N ha<sup>-1</sup>). At the improved site, the mean urine N loading rates were significantly lower (t-test;  $n = 97$ ;  $p < 0.05$ ) ( $621 \pm 22$  kg N ha<sup>-1</sup>) compared to the semi-improved site in autumn ( $966 \pm 63$  kg N ha<sup>-1</sup>), 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<sup>2</sup> wetted area, or 5 L m<sup>-2</sup>). 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<sup>-2</sup> at the semi-improved site and 8.9 L urine m<sup>-2</sup> 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<sup>-1</sup> for dairy cattle urine patches and 500 kg N ha<sup>-1</sup> 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<sup>-1</sup>). The mean urine patch N loading rate across all trials was  $838 \pm 31$  kg N ha<sup>-1</sup>, which is higher than that reported by Haynes and Williams (1993). This suggests that the N loading rates and subsequent estimates of NH<sub>3</sub> volatilisation, N<sub>2</sub>O emissions and NO<sub>3</sub><sup>-</sup> 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<sub>2</sub>O 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



**Fig. 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,  $\text{NO}_3^-$ -N data were omitted as values were negligible. Stacked bars represent the mean values for each season and site; legend applies to both panels.

**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
$\text{KHCO}_3$ ( $\text{g L}^{-1}$ )	6.0	6.5
KCl ( $\text{g L}^{-1}$ )	3.5	4.0
$\text{Na}_2\text{SO}_4$ ( $\text{g L}^{-1}$ )	0.4	3.0
$\text{CaCl}_2$ ( $\text{g L}^{-1}$ )	–	0.1
Urea ( $\text{g L}^{-1}$ )	6.4	6.5
Creatine ( $\text{g L}^{-1}$ )	0.85	0.85 <sup>a</sup>
Hippuric acid ( $\text{g L}^{-1}$ )	1.85	4.4
Allantoin ( $\text{g L}^{-1}$ )	0.6	1.5
Glycine ( $\text{g L}^{-1}$ )	0.01	0.01
Creatinine ( $\text{g L}^{-1}$ )	0.015	0.3
Uric acid ( $\text{g L}^{-1}$ )	0.005	0.1
Hypoxanthine ( $\text{g L}^{-1}$ )	0.001	0.001 <sup>a</sup>
Ammonium chloride ( $\text{g L}^{-1}$ )	0.015	0.3
Total N content ( $\text{g N L}^{-1}$ )	3.6 <sup>b</sup>	4.4 <sup>b</sup>

<sup>a</sup> Creatine and hypoxanthine were not measured in the current study.

<sup>b</sup> Note if higher N concentrations are required for experimental purposes we recommend increasing the amount of urea as desired.

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 ( $1.2\text{--}13 \text{ g N L}^{-1}$ ) are fairly consistent with other data reported in the literature. For example, Bristow et al. (1992) observed urine N contents between 3 and  $13.7 \text{ g N L}^{-1}$  in sheep fed a ryegrass/white clover pasture; Hoogendoorn et al. (2010) reported a range of  $0.5\text{--}16.6 \text{ g N kg}^{-1}$  in sheep grazing a common ryegrass/cocksfoot/white clover pasture and Doak (1952) reported sheep urine N concentrations between 5.7 and  $12 \text{ g N L}^{-1}$ .

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) (2.2–11.8 % of total N). Hippuric acid, which is derived from the breakdown of phenolic compounds, comprised the next largest N fraction (0.2–34 % of urine-N). This compares with 2.6–7.1 % reported by Bristow et al. (1992) for sheep. Creatinine, formed via degradation of creatine and creatine-phosphate (Dijkstra et al., 2013) comprised 0.1–7.3 % of the urine N content.

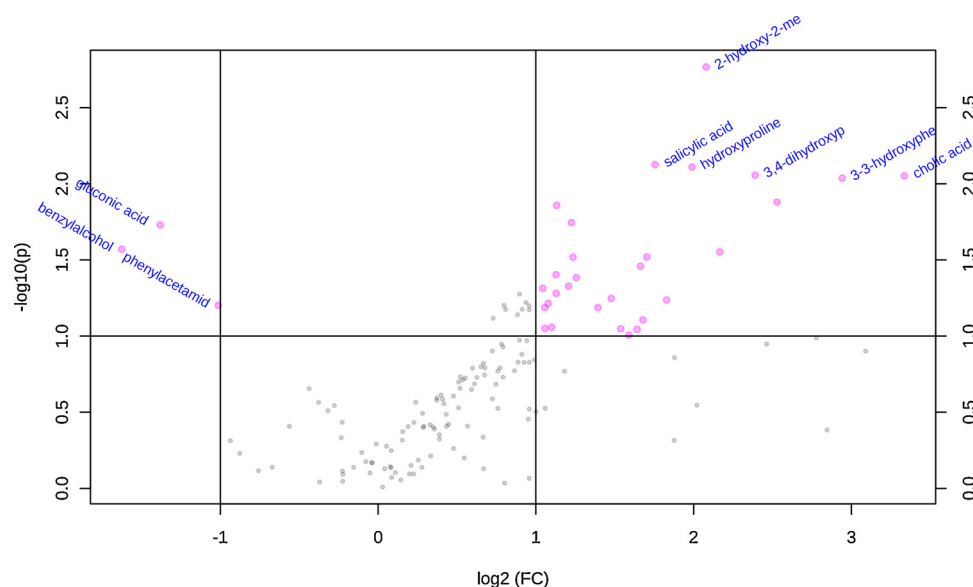
All other nitrogenous urine constituents analysed made up less than 1 % of the total N, on average. The variations in average  $\text{NH}_4^+$  concentrations (principally a product of urea hydrolysis) may have been due to differences in sample transport time to the cold store but also due to cross-reactivity by organic N during sample analysis (Herrmann et al., 2005). Free amino acids were a much smaller fraction of the total N content in this study (< 1%) than the fractions reported by Doak (1952) and Bathurst (1952) for sheep, which ranged between 9.3 and 15.9 % of the urine-N content. The disparity in the values measured for the amino acid fraction could be due to improvements in specificity of more recent methods to measure amino acids. The greater ranges in urine-N constituents reported in this study compared to others reflects the larger sample sizes used e.g. analysing nearly 200 individual urine events compared to e.g. five individual sheep urine events in Bristow et al. (1992), one event in Bathurst (1952) and 12 events in Doak (1952).

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

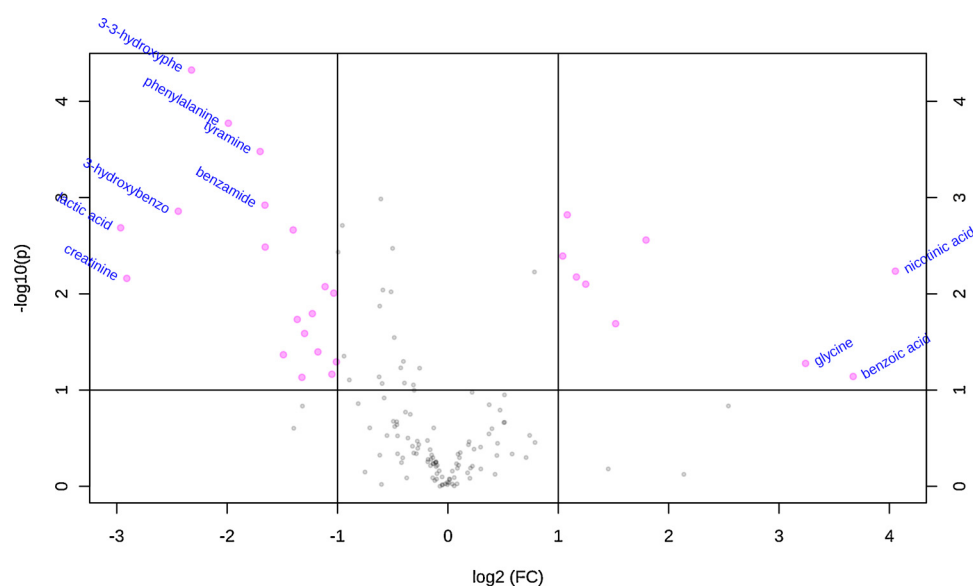
### 3.5. Artificial urine recipe

Utilising the urine chemical composition data from this study we provide an artificial urine ‘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





**Fig. 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.



**Fig. 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.

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<sub>2</sub>O 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.

#### Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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#### Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:<https://doi.org/10.1016/j.agee.2020.107073>.

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