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Assessment of water balance and body water dynamics in a temperate sheep breed using ^{2}H-labelled drinking water

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ABSTRACT

Water availability is a growing concern for livestock farmers due to climate change, particularly in arid regions and increasingly in temperate regions. Water use efficiency may become an important trait to select for in temperate breeds, thus the objective here was to evaluate the water balance and body water dynamics of Lleyn ewes. Ewes (n = 15), assigned to one of three isotopically distinct drinking waters (DW) with δ^{2}H values of −49‰, + 241‰ and + 1143‰, were housed individually and fed at maintenance for 82 days. Total water (TW) intake was recorded daily and water loss *via* urine and faeces was determined daily during four campaign weeks. The average TW intake was 2.7 ± 0.63 kg d^{-1} with DW contributing 86.3 ± 1.98%. There was a strong correlation between DW intake and temperature-humidity index (r = 0.77; $p < 0.01$). The average measured water loss was 39.4% of TW intake, indicating a large unmeasured route of water loss, likely expiration. Time-series isotopic analysis of blood and urine waters revealed average half-lives of 13.2 ± 4.09 d and 14.4 ± 5.18 d, respectively, and they were not statistically different. There was a three-fold variation in half-lives among ewes, demonstrating that there is potential to increase the water use efficiency in temperate sheep breeds, aligned to future breeding goals to improve sustainability traits. An isotope mixing model was established to predict equilibrium body water δ^{2}H values, but future work is required to refine the model to account for ^{2}H incorporation into body tissues.

1. Introduction

Increasing temperatures, water shortages, reduced water quality, and poor water distribution due to climate change pose a significant threat to livestock farming (Akinmoladun et al., 2019; IPCC, 2019). The production of feed makes up the majority of water use in the ruminant sector, *via* irrigation and rainfall (Beede, 2012; Legesse et al., 2017). However, reduced supply of drinking water (DW), particularly in arid regions and increasingly in temperate regions (Schütz et al., 2024), compounded by increased DW requirements due to heat stress, mean that climate change is having direct impacts on animal welfare and productivity (Beede, 2012; IPCC, 2019). In growing sheep (*Ovis aries*), restricted DW can reduce feed intake and weight gain (de Souza et al., 2022). On the other hand, increased water intake in goats (*Capra hircus*), as a response to heat stress, has been demonstrated to reduce the protein content, and therefore product quality, of milk, possibly as a result of reduced microbial protein synthesis in the rumen due to changes in rumen digestion kinetics (Hamzaoui et al., 2013).

Nevertheless, small ruminants have lower water requirements than cattle (*Bos sp.*), both indirectly to produce feed and for direct consumption, and they also tend to cope better in hot and arid climates (Silanikove, 2000; Eisler et al., 2014; Joy et al., 2020; Pulido-Rodríguez et al., 2021). These characteristics mean that small ruminants will likely become more important in global agriculture (Beede, 2012; Eisler et al., 2014). Among small ruminant species, sheep tend to have greater water requirements than goats, with one study finding 70% higher total water

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(TW) intake per unit metabolic weight in sheep (Al-Ramamneh et al., 2010). It is well established that lactation increases water intake (Calianno et al., 2025), and that season (Longhurst et al., 1970) and animal management such as shearing (Al-Ramamneh et al., 2011; Calianno et al., 2025) and walking distance to water (Squires and Wilson, 1971) affect water intakes in both sheep and goats. Furthermore, there are marked differences in water requirements between breeds, largely due to selective breeding to suit hot and arid climates. For example, in a South African climate, Blackhead Persian sheep, which have been specifically bred to suit a hot and arid climate, consume significantly less water than Merino and Dorper sheep (Schoeman and Visser, 1995).

Several studies have found that small ruminants are able to withstand periods of water restriction, particularly breeds suited to arid environments (Jaber et al., 2004; Al-Ramamneh et al., 2012; Pahlane et al., 2025). Nevertheless, offering water *ad libitum* may be necessary to safeguard animal welfare (Jensen and Vestergaard, 2021). Furthermore, more extreme environmental conditions and heat stress are still major concerns to small ruminant welfare and productivity globally (Marai et al., 2007; Hamzaoui et al., 2013; Joy et al., 2020). Understanding the water requirements and water use efficiency of small ruminants, particularly in breeds suited to temperate climates where there has been less research, is therefore essential to improve animal health and welfare, support farmer livelihoods and global food security (Akinmoladun et al., 2019).

Body water half-life is an indicator of water use efficiency, whereby longer half-lives signify higher water use efficiency. Body water turnover and half-life in terrestrial animals is typically determined using an isotope tracer (deuterium ^{2}H or tritium ^{3}H) by injecting a known dose of labelled water and monitoring the depletion of the tracer in body water samples, usually blood, over time. Previous isotope dosing studies in sheep have found a wide range of body water half-lives from 2.3 days to 16.3 days with factors including breed, ambient conditions and animal management having an effect (Till and Downes, 1962; Anand and Parker, 1966; Longhurst et al., 1970; Faichney and Boston, 1985). The ^{2}H dilution technique, whereby the concentration of ^{2}H in blood plasma is measured following administration and equilibration of a measured dose of labelled water, has also been used to confirm higher water intakes in sheep than goats (Al-Ramamneh et al., 2010) and increased DW intake in unshorn sheep compared to shorn (Al-Ramamneh et al., 2011).

A limitation of dosing with isotope tracers to estimate body water dynamics is that incorporation of ^{2}H or ^{3}H into body tissues *via* H exchange and during tissue biosynthesis means that experimentally determined turnover rates are higher than actual turnover rates (Streit, 1982). Feeding at maintenance to limit tissue biosynthesis can minimise incorporation of the tracer into tissues. An alternative approach to observing the elimination of a tracer from the body following a single dose is to monitor its incorporation by administering the tracer over a long period of time.

As such, the objectives of this study were to carry out a DW-switch using ^{2}H-labelled DW to determine: (1) the water balance of Lleyn ewes, a sheep breed of increasing popularity in temperate regions such as the UK, when fed at maintenance; and (2) the variation in body water half-life, and therefore water use efficiency, among individuals from the same flock. It was hypothesised that there would be differences in water use efficiency among ewes raised in the same flock, presenting a potential for selective breeding to improve water use efficiency of temperate sheep breeds.

2. Materials and methods

2.1. Ethical approval

The experiment was carried out at Harper Adams University, UK under the auspices of the UK Home Office License PPL P84F49BB5 as regulated by the Animals (Scientific Procedures) Act 1986 and amended by the Animals (Scientific Procedures) Act 1986 Amendment Regulations 2012. The protocol was approved by the Animal Welfare and Ethical Review Board of Harper Adams University.

2.2. Animals and experimental design

The DW-switch experiment lasted for 82 days, with a 7-day acclimatisation period beforehand, from June to September 2022 at Harper Adams University's Future Farm. Lleyn ewes ($n = 15$), aged 5–7 years and raised since birth at Harper Adams University, were housed indoors in individual pens in the same experimental barn during the acclimatisation period and throughout the experiment. The ewes were shorn before the acclimatisation period. Ewes were fed at maintenance on a commercially available dried grass feed (Graze-on Original, Northern Crop Driers, York, UK) comprising *Lolium* and *Festulolium* species, and constituting 93% dry matter, 14% crude protein, 3.5% oil, 25% fibre, 12% sugar and 1.5% starch as stated on the feed label. Energy requirements for maintenance were calculated according to AFRC (1993), with an additional 5% added as a safety margin (AFRC, 1993). The ewes were weighed weekly. The average liveweight (LW) at the start of the acclimatisation (Week 0) and Week 1 was 84.9 ± 9.94 kg and 80.9 ± 10.35 kg, respectively. The LW at the start of Week 1 was used as the benchmark for the experiment and feed allowances were adjusted accordingly with a 5% decrease or increase in LW compared to the benchmark warranting a 10% increase or decrease in feed allowance, respectively. The average feed allowance across the experiment was 0.97 ± 0.094 kg d^{-1} and the ration was fed in two halves at 08:00 h and 17:00 h.

Stable isotopic compositions are given as delta (δ) values, expressed as permille (‰), relative to an international standard (Hayes, 2004); Eq. 1). During the acclimatisation period, all ewes had *ad libitum* access to DW from an on-site borehole with a $\delta^{2}H$ value of −49‰ (hereafter referred to as 'Tap' water). Prior to the DW switch, the ewes were assigned to either the Tap DW or one of two ^{2}H-enriched DW with $\delta^{2}H$ values of + 241‰ (hereafter referred to as '+250') and + 1143‰ (hereafter referred to as '+1000') in a randomised block design according to LW, such that there were three treatment groups ($n = 5$ per group). This study is part of a wider study investigating the routing of water-derived H atoms from DW to ruminant tissues, thus the sample size was determined by conducting a power analysis based on the biological variability of adipose fatty acid $\delta^{2}H$ values. The DW was switched at 08:00 h on Day 1. The absolute ^{2}H concentration of the + 1000 DW is 334 ppm, compared to 148 ppm for the Tap DW. The threshold for adverse effects is 10–20% $^{2}H_2O$, thus physiological effects are not expected at such low ^{2}H concentrations (Koletzko et al., 1997).

$$\delta^{2}H = \left(\frac{R_{sample}}{R_{VSMOW}} - 1\right) \times 10^{3} \quad (1)$$

Where R values are the absolute $^{2}H/^{1}H$ ratios of the sample and the VSMOW international standard (= 1/6419; (Hayes, 2004).

Weeks 1, 4, 8 and 12 were campaign weeks, during which sheep were moved to individual collection pens with raised slatted floors so that urine and faecal output could be collected. Blood sampling occurred during the acclimatisation week and campaign weeks. During the acclimatisation week and the non-campaign weeks, the ewes were housed in individual standard pens on sawdust bedding. The ewes were transferred from the standard pens to the collection pens at 08:00 h on the first day of each campaign week, and back to the standard pens at 08:00 h on the first day of the following week.

2.2.1. Preparation of isotopically enriched drinking waters

The + 250 DW and + 1000 DW were prepared by mixing Tap water and a 'stock' water with a $\delta^{2}H$ value of approximately + 4000‰. All drinking waters and the stock water were made up and stored in sealed 1000 L industrial bulk containers.

The stock water was prepared by mixing $^{2}H_2O$ (99.9%; *ca.* 630 mL;

Sigma-Aldrich) with Tap DW (*ca.* 1000 L). The + 250 DW was made by mixing stock water (*ca.* 74 L) with Tap DW (*ca.* 926 L) and the + 1000 DW was made by mixing stock water (*ca.* 740 L) with Tap DW (*ca.* 260 L). The DW were remade once during Week 8 of the experiment to ensure that there were sufficient quantities. The δ^2H values of the two batches were then measured and the average δ^2H values (Table 1) were used in all analyses.

2.2.2. Total water Intake

Total water (TW) intake is defined as the sum of DW intake, metabolic water produced from feed digestion and intake of feed moisture.

Every pen had a self-filling water bowl with a float valve mechanism attached *via* hosing to a water drum fixed to the front hurdle of the pen, which allowed assessment of different experimental waters. The drums were closed but not sealed with a lid to minimise evaporation and resulting isotopic fractionation. Individual DW intake was measured daily at 08:00 h. The water drums were emptied into a bucket, which was subsequently weighed and topped up to *ca.* 5 L with the appropriate DW and weighed again. The water was transferred back to the drum. DW intakes were determined by subtracting the pre-topped-up weight from the previous day's topped up weight.

Metabolic water produced from the oxidation of feed was calculated according to the composition of the feed and the water yield from the oxidation of each component (Maynard, 1979). Johnson and Ward (1996) reported a range of 2–12% loss of energy as methane related to the diet with higher forage diets resulting in a greater loss of energy. An adjustment of 8%, at the higher end of the range due to nature of our restricted diet, was made to account for the loss of energy as methane. Therefore, 1 kg of fresh feed was estimated to yield 0.28 kg of metabolic water (Table 1).

Feed samples were taken during each campaign week and freeze-dried to determine the moisture content. The water intake from feed moisture was calculated as the product of average moisture content and daily feed intake.

2.2.3. Collection of urine and faeces

The pens used during the campaign weeks had slatted flooring, allowing urine and faeces to pass through. Below the slatted flooring was a stainless-steel mesh (1 mm hole size) to separate the faeces from the urine. The mesh could be pulled out to collect the faeces. A urine tray was placed underneath the mesh at an angle, allowing the urine to run into the collection bowl underneath.

Urine and faeces were collected and weighed daily during the campaign weeks at 08:00 h. Faeces, 10% of total weight, were collected and stored at −18 °C. The water content of the faeces was determined by freeze-drying, thus water loss in the faeces is the product of faecal output and moisture content. Urine samples (two *ca.* 5 mL aliquots) were collected as soon after the first urination event of the day as possible (typically before 12:00 h, or 17:00 h on occasions when the ewe did not urinate earlier in the day) to limit isotopic fractionation associated with evaporation, and stored at −18 °C prior to extracting the water for isotopic analysis.

2.2.4. Blood sampling

Blood sampling occurred by jugular venepuncture on the final day of the acclimatisation week, and once towards the end of each campaign week, by a competent Home Office Personal license holder. Blood sampling was always carried out at 12:00 h, approximately 4 h after morning feeding. A total of 50 mL was collected with 40 mL in additive-free vacutainers and 10 mL in Li^+-heparin vacutainers. The vacutainers were stored at 4 °C until processing later the same day.

Serum was collected from the additive-free vacutainers after the blood had coagulated. The Li^+-heparin vacutainers were spun in a centrifuge at 1000 *g* for 15 min before the plasma was collected. The serum was stored −18 °C prior to sample preparation for future fatty acid isotopic analysis.

Table 1
Measured δ^2H values of the three drinking waters for Batch 1 and Batch 2, and the average of Batches 1 and 2.

	δ^2H value [SD] (‰)		
Drinking water	Batch 1	Batch 2	Average
Tap	-52 [2.2]	-46 [4.4]	-49 ± 2.4
+ 250	239 [1.9]	243 [1.7]	241 ± 1.3
+ 1000	1132 [4.8]	1152 [3.8]	1143 ± 3.1

Table 2
Composition of experiment feed and corresponding metabolic water yield. Methane adjusted total assumes that 8% of the energy is lost as methane. Values of metabolic water produced from each feed component are from Maynard (1979).

Component	Amount in 1 kg fresh feed (kg)	Metabolic water per kg component (kg)	Metabolic water yield per kg fresh feed (kg)
Carbohydrate	0.385	0.56	0.216
Protein	0.140	0.40	0.056
Fat	0.035	1.07	0.037
Total			0.309
Methane-adjusted total			0.284

2.2.5. Rumen fluid sampling

The ewes were electrically stunned and euthanised, by exsanguination of the carotid artery, at the end of the study period. Rumen fluid was collected from each ewe by filtering rumen digesta through two layers of muslin cloth. Approximately 20 mL of rumen fluid was collected for water isotopic analysis and stored at −18 °C until water extraction for isotopic analysis.

2.3. Temperature-humidity index

A TinyTag data logger (Gemini Data Loggers, Chichester, UK) was placed near the centre of the experimental barn to record the temperature and relative humidity every 15 min. The average daily temperature-humidity index (THI) was calculated using Eq. 2 according to LPHSI (1990) and modified by Marai et al. (2001).

$$THI = T - [(0.31 - 0.31RH)(T - 14.4)] \quad (2)$$

Where *THI* is the temperature-humidity index, *T* is the dry bulb temperature (°C) and *RH* is the relative humidity (%/100)

2.4. Water isotopic analysis

Water in the blood serum, urine and rumen fluid was extracted by cryogenic vacuum distillation using a modified version of the protocol described by IAEA (2016). Approximately 2 mL of the sample was extracted. Samples were frozen at −18 °C and, immediately prior to evacuation, a piece of glass wool was placed in the sample tube to prevent the sample from bubbling into the collection tube upon later heating. The sample tube and trapping tubes were connected *via* a glass tube and the system was evacuated, keeping the sample frozen using liquid nitrogen. Once evacuated, the sample was heated to 80 °C (rather than 100 °C as specified in IAEA (2016), to prevent vigorous bubbling), and the collecting tube was held in liquid nitrogen. Evaporated water was condensed and frozen using liquid nitrogen in the collecting tube. Once all the water was extracted, the thawed extracted water was transferred to a vial, leaving no headspace. Extracted waters were stored at 4 °C for *ca.* 1 week until isotopic analysis.

The isotopic compositions of the three DW and the extracted waters were measured by Off-Axis Integrated Cavity Output Spectroscopy (OA-ICOS) using a Los Gatos Research Triple-Liquid Water Isotope Analyser

(T-LWIA; model 912–0050). Samples (950 nL) were introduced into the OA-ICOS *via* a PAL LC-xt autosampler and injected into a heated injector block (LGR) using a Hamilton 1.2 uL syringe (7701.2 KH CTC). Samples were vapourised to 100 °C and the cavity held at 45 °C. Each sample was injected 6 times and the average measured values were used. The average SD was 1.76‰. Four preparative injections between samples prevented carryover. The LWIA Post Analysis software version 4.5 was used to process the data, using inter-run standard measurements (ABB-LGR, part SPK-WIA-WS-ERS) as a calibration to correct the raw values. The standards used for the calibration are displayed in Table 3.

2.5. Body water dynamics

An exponential one-compartment model (Eq. 3), as used by Harrison et al. (2011) and adapted from Hesslein et al. (1993), was used to fit the time-series water isotopic data from the + 250 and + 1000 groups. The half-lives of blood water and urine water were then calculated according to Eq. 4.

$$C_t = C_E + (C_0 - C_E) \times \exp(a * t) \quad (3)$$

Where C_t is the isotopic composition at time t; C_E is the isotopic composition at equilibrium; C_0 is the initial isotopic composition; and a is the turnover constant.

$$t_{1/2} = \frac{\ln(2)}{a} \quad (4)$$

2.6. Normalising for variance between groups

Where data is pooled from multiple DW groups, the data was normalised for different levels of variance between the groups according to Eq. 5.

$$\delta^2 H_{norm} = \frac{\delta^2 H_{raw} - \delta^2 H_{ewe\ mean}}{\delta^2 H_{group\ SD}} \quad (5)$$

Where, for each measurement, $\delta^2 H_{norm}$ is the normalised δ^2H value; $\delta^2 H_{raw}$ is the measured δ^2H value of blood water, rumen water or urine water; $\delta^2 H_{ewe\ mean}$ is the mean δ^2H value of blood water, rumen water and urine water from the individual ewe; $\delta^2 H_{group\ SD}$ is the standard deviation of all water pool measurements from all ewes in the respective DW group.

2.7. Statistical analysis

All statistical analyses were performed using SPSS 28.0.1.1 software. Non-parametric tests were used as normality could not be reliable confirmed. Mann-Whitney tests were used for comparisons between DW groups and Wilcoxon signed-rank rests were used for comparisons within DW groups. Regression analyses were performed in Origin 2024b. Outliers were identified as points with a Studentized residual value greater than 2.The significance level was set at $p < 0.05$ for all statistical analyses. Where datapoints were missing, data from that individual was excluded from statistical tests.

Table 3
Standard used for the calibration of measured δ^2H values of water samples. δ^2H values are relative to VSMOW.

Working standard	δ^2H value [standard deviation] (‰)
#ER1A	107.2 [1.0]
ER2A	192.4 [1.0]
#ER3A	381.0 [1.5]
#ER4A	642.4 [1.5]
ER5A	860.1 [2.0]

3. Results and Discussion

3.1. Sheep liveweights

The average LW at the start of the acclimatisation (Week 0) was 84.9 ± 9.94 kg. The average LW loss during the acclimatisation week was 4.8 ± 2.60% such that the mean LW was 80.9 ± 10.35 kg at the start of Week 1 (Fig. 1). This decrease in was likely due to a decrease in gut fill, which is *ca.* 10% of LW (Litherland et al., 2010), as the ewes were moved from an *ad libitum* fresh pasture diet to being fed chopped dried grass at maintenance. For most ewes, LW change after the acclimatisation period was not substantial enough to warrant a change in feed allowance. From Week 6 onwards, there were no changes to feed allowance, although there were small increases in the average LW. The mean average LW at the end of the experiment was 80.9 ± 10.18 kg (Fig. 1).

3.2. Water balance

3.2.1. Total water intake

The average daily water intakes from DW, feed moisture and metabolic water (digestion of feed) were 2.4 ± 0.61 kg, 0.1 ± 0.01 kg and 0.3 ± 0.03 kg, respectively. Metabolic water is also produced during anabolic metabolism but, since the ewes were fed at maintenance such that their LW did not change significantly during the experiment (Fig. 1), this was not considered as a source of water for balance purposes. The average TW intake was therefore 2.7 ± 0.63 kg d^{-1}, with DW, feed moisture and metabolic water contributing 86.3 ± 1.98%, 2.5 ± 0.36% and 11.2 ± 1.61%, respectively. This agrees with the results of Wallace et al. (1972) who studied the water intakes in sheep on a dry diet and concluded that DW, feed moisture and metabolic water contribute 88–90%, 1.9–2.9% and 7.2–10.1%, respectively. The average DW intake per unit metabolic weight ($LW^{0.75}$) was 89 ± 18.6 g d^{-1} and TW intake/$LW^{0.75}$ was 102 ± 19.0 g d^{-1}. A Kruskal-Wallis test with Bonferroni correction confirmed that there was no difference in TW intake/$LW^{0.75}$ between the Tap and + 250 DW groups (92 ± 11.8 g d^{-1} and 94 ± 8.7 g d^{-1}, respectively), but it was greater ($p < 0.05$) in the + 1000 group (120 ± 21.0 g d^{-1}) compared to the Tap group. There is a weak positive correlation between TW intake and metabolic weight ($r = 0.3$) and DW intake and metabolic weight ($r = 0.02$), but the slopes are not significant ($p > 0.05$; Fig. 2).

The greater TW intake in the + 1000 group was unexpected and raises the question of whether ^{2}H enrichment could have affected DW palatability or drinking behaviour. For humans, highly pure ^2H_2O does

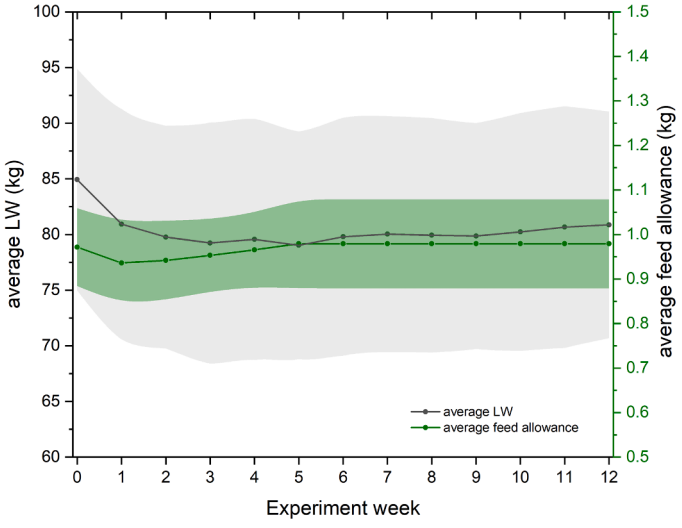

Fig. 1. Average liveweight (LW; grey) and average daily feed allowance (green) over the time course of the experiment. Shaded areas represent 1 SD.

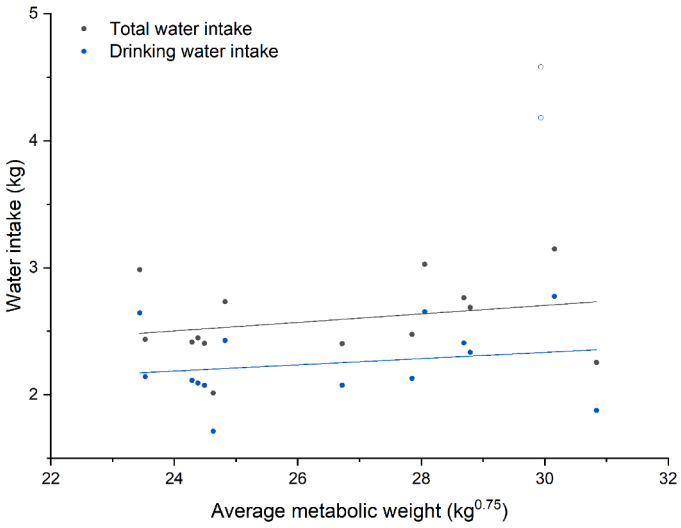

Fig. 2. Total water intake (black) and drinking water intake (blue) against average metabolic weight. Regression lines exclude the outlier ewe (open datapoints); r = 0.3 for TW intake and 0.2 for DW intake; $p > 0.05$.

taste sweeter compared to $^{1}H_2O$, but this is not true for mice (Ben Abu et al., 2021) and, as far as the authors are aware, this has not been investigated in sheep. However, the ^{2}H concentrations used in the current experiment are 13 orders of magnitude smaller than in 99% pure $^{2}H_2O$, thus it is highly unlikely that ^{2}H enrichment affected DW palatability.

The absolute intakes from feed moisture and metabolic water did not vary significantly over the experiment timeframe since they are dependent on feed allowances, which largely remained constant due to maintenance feeding (Fig. 1). There were large diurnal fluctuations in DW intake aligned with fluctuations in the THI (Fig. 3a). There was a strong positive correlation ($r = 0.77$; $p < 0.01$) between DW intake and THI, and it is likely that the sheep experienced some heat stress during several days of the experiment, which could increase water turnover rate (THI > 22.2; Fig. 3b). There was no trend in DW intake when adjusted for THI during the acclimatisation period and experiment (Fig. 3c).

Despite periods of high THI, DW intake/LW$^{0.75}$ (92 – 120 g d^{-1}) was lower in the current study compared to earlier studies. For example, DW intake/LW$^{0.75}$ found by Sunagawa et al. (2001) for crossbred Merino ewes was 131 – 161 g d^{-1} and Al-Ramamneh et al. (2011) found similar (134 – 165 g d^{-1}) in German Blackhead sheep. Till and Downes (1962) found DW intake/LW for Merino wethers was ca. 60 g kg^{-1} d^{-1}, compared to 24 – 47 g kg^{-1} d^{-1} in the current study. The difference in results between the present study and these studies could be due to differences in breed characteristics, such as the selection for wool production in Merino sheep resulting in increased energy metabolism and consequently increased water turnover (NRC, 2007). It is also possible that the ewes in the present study were not fully acclimatised to the experimental conditions (i.e. drinking from a water bowl), which affected their drinking behaviour (Teixeira et al., 2006; Coimbra et al., 2010; Ferreira et al., 2025). However, in this case, it would be expected that the average DW intake would increase over the course of the experiment as the ewes became acclimatised, whereas there was no such trend in DW intake during the experiment (Fig. 3c). A more likely explanation for the lower water intake compared to those in the literature is that in other studies, the sheep were fed *ad libitum*, rather than at maintenance as they were in the current study. As demonstrated by Forbes (1968), there is a positive linear correlation between TW intake and dry matter intake in non-lactating and non-pregnant sheep, thus a higher dry matter intake with *ad libitum* feeding will likely result in a higher DW intake. TW intake against dry matter intake in the current study (Fig. 4) lies within the uncertainty of the relationship between DMI and TWI established by Forbes (1968), supporting this assertion.

3.2.2. Measured water loss

The measured routes of water loss were urinary water and faecal water. The urine water was consistent across the four campaign weeks, with an average of 1.0 ± 0.55 kg d^{-1}, 1.1 ± 0.55 kg d^{-1}, 1.0 ± 0.57 kg d^{-1} and 0.9 ± 0.42 kg d^{-1} for Weeks 1, 4, 8 and 12, respectively. Faecal output (fresh weight) was also consistent across the four campaign weeks, with an average of 0.33 ± 0.109 kg d^{-1}, 0.29 ± 0.060 kg d^{-1}, 0.29 ± 0.090 kg d^{-1} and 0.29 ± 0.114 kg d^{-1} for weeks 1, 4, 8 and 12, respectively. There was more variation in faecal water content, both between the campaign weeks and between individuals, with average values of 29.5 ± 11.19%, 27.0 ± 5.23%, 30.0 ± 7.90% and 36.7 ± 8.07% for weeks 1, 4, 8 and 12, respectively. These values correspond to an average faecal water loss of 0.09 ± 0.018 kg d^{-1}. Urine water and faecal water therefore contributed 90.8 and 9.2 ± 2.72%, respectively, to measured water output.

There was a strong positive correlation between water output and TW intake ($r = 0.94$; $p < 0.01$). This was driven by urine water ($r = 0.94$; $p < 0.01$). There was a weak correlation between faecal water and TW intake ($r = 0.39$) but this was not significant ($p = 0.29$; Fig. 5). These results are consistent with the fact that the kidneys are the main organ responsible for the regulation water balance (Skotnicka et al., 2007), thus urine volume is more sensitive to TW intake than faecal moisture.

3.2.3. Water balance model

Based on the measurements and calculations of water intake and loss, a model to describe the water balance in the ewes was constructed (Fig. 6). Inhaled vapour is also recognised as a possible water source, but it was not measured and it is likely minor as has been determined in cetaceans (< 5%) using isotope box modelling (Séon et al., 2023).

Measured water output (urine water + faecal water) was 39.4 ± 6.59% of determined TW intake, indicating that there is a large unmeasured water loss (Fig. 6). Water is also lost through vapour, primarily from the lungs as expired air, during rumination and, to a limited extent in sheep, through sweat (Church, 1993). These findings are consistent with earlier studies. For example, Anand and Parker (1966) found that water loss in urine was 37% of the TW intake and Wallace et al. (1972) found that vaporised water was 53% of the total water loss in sheep maintained indoors at 20–26 °C. These conditions were comparable to those in the current experiment thus the higher proportion determined in the current experiment ('Other water loss' in Fig. 6) could be because faecal water is relatively low on account of the dry feed used in this study. Future studies entailing the use of respiratory chambers or 'doubly labelled water' could be conducted to directly or indirectly quantify water loss through expiration (Midwood et al., 1994).

3.3. Body water dynamics

It was hypothesised that the half-lives of blood water and urine water would be similar since blood water is the source of urine water (Michalek, 2018). Furthermore, since half-life is independent of the initial isotopic composition (C_0) and isotopic composition at equilibrium (C_E; Eq. 4), it was expected that the half-life would be the same for the + 250 and + 1000 groups.

The H isotopic compositions ($\delta^{2}H$ values) of blood water (Fig. 7a) and urine water (Fig. 7b) reached equilibrium (plateaued) with the DW during the timeframe of the experiment. The average half-lives of blood water and urine water across both the ^{2}H-enriched DW groups (+250 and +1000) were 13.2 ± 4.09 days and 14.4 ± 5.18 days, respectively, and are not statistically different (Wilcoxon Signed Rank test; $p > 0.05$). The average half-lives within the + 250 and + 1000 groups are displayed in Table 4. There is no difference in the half-lives of blood water and urine water in the + 250 group ($p > 0.05$) but in the + 1000 group the half-life of urine water is longer than blood water for all ewes

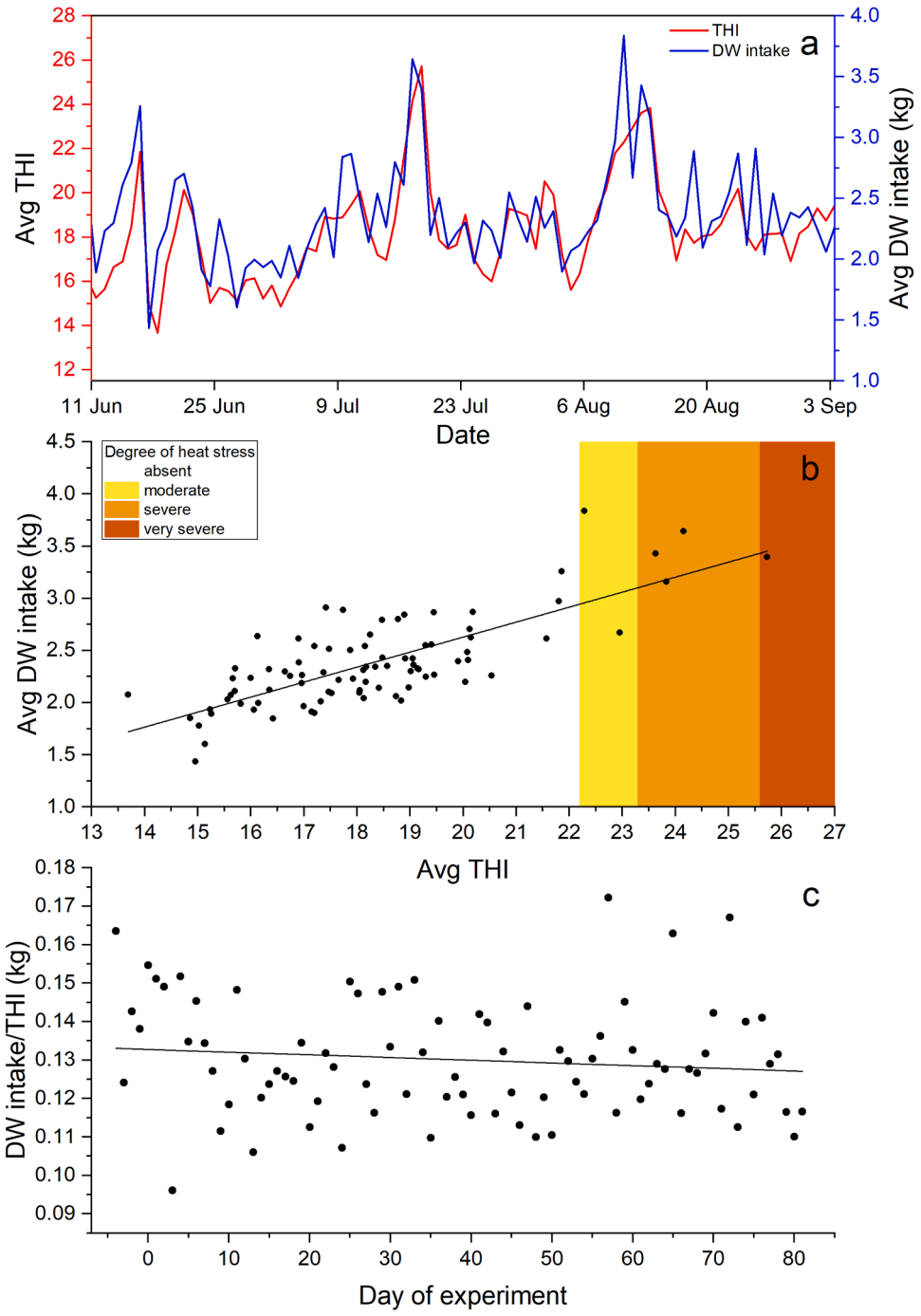

Fig. 3. Relationship between drinking water (DW) intake and temperature-humidity index (THI). a) Daily average THI (red) and average DW intake (blue). b) Average DW intake against average THI. Boundaries of degrees of heat stress according to LPHSI (1990) and modified by Marai et al. (2001). $r = 0.77$; $p < 0.01$. c) DW intake adjusted for THI during the acclimatisation period (day of experiment ≤ 0) and experiment. The slope of the regression line is not significant ($p > 0.05$; $r = -0.1$).

(Wilcoxon Signed Rank test; $p = 0.043$). The half-lives are shorter in the + 1000 group compared to the + 250 group (Table 4), but this is not significant (Mann-Whitney test; $p = 0.056$ for blood water and $p = 0.421$ for urine water).

When the half-life of urine water is plotted against half-life of blood water for individual ewes, the slope and intercept are close to 1 and 0, respectively, confirming that the half-lives of urine water and blood water are the same (Fig. 8a). There is a negative correlation ($r = -0.8$; $p = 0.01$) between average half-life of blood water and urine water and TW intake normalised by metabolic weight (Fig. 8b). The ewes in the + 1000 group tended to have a higher TW intake/$LW^{0.75}$ than those in the + 250 group (Fig. 8b), and this could account for the faster (but not statistically significant) half-lives in the + 1000 group compared to the + 250 group. After adjusting for TW intake/$LW^{0.75}$ ANCOVA, DW group did not predict half-life ($p = 0.219$) but TW intake/$LW^{0.75}$ showed a trend with half-life ($p = 0.057$). The three-fold variation in half-life (Fig. 8b) suggests that there is a wide range in water use efficiency among the ewes.

Although it is recognised that there are several water compartments in sheep (e.g. rumen water, blood plasma, intracellular water) with

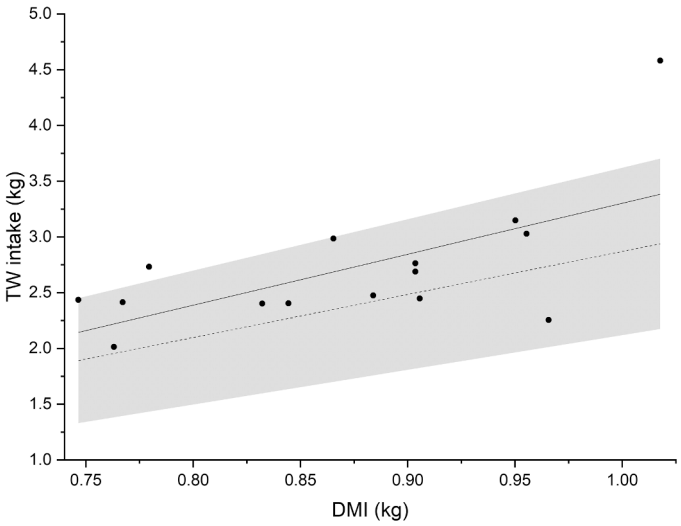

Fig. 4. Total water (TW) intake against dry matter intake (DMI). Datapoints are daily average intakes for each ewe, and the solid line is the regression (slope = 4.6 ± 1.56; r = 0.6, p < 0.05; intercept = −1.3 ± 1.37). The dashed regression line is the relationship between DMI and TWI established by Forbes (1968) and the shaded area is the uncertainty of the slope.

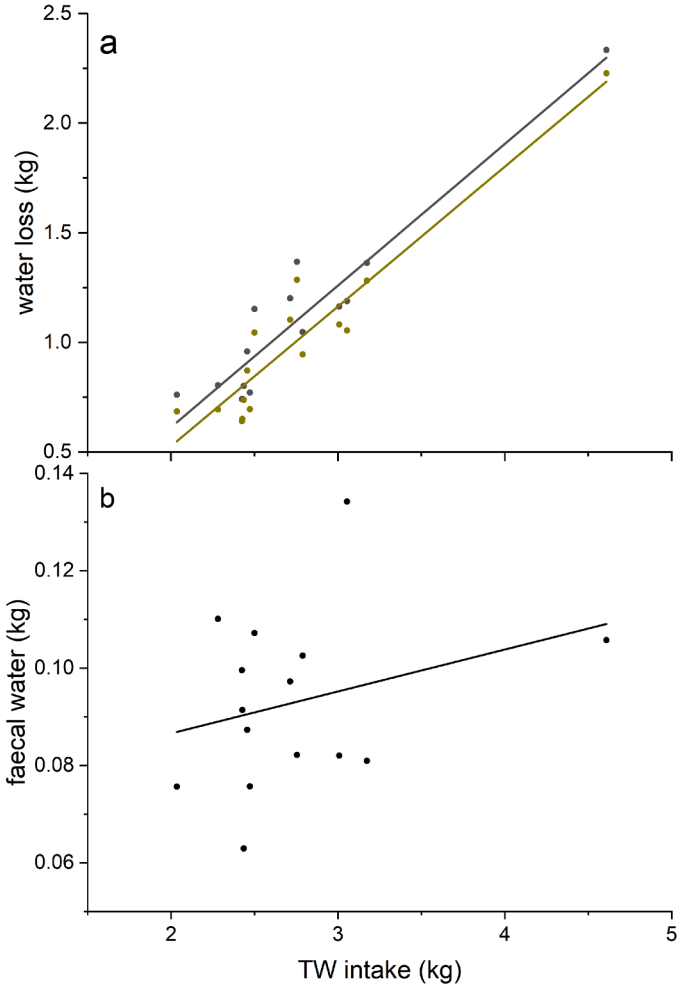

Fig. 5. Water loss against total water (TW) intake. a) Total measured losses, black (r = 0.94; p < 0.01); urinary losses, dark yellow (r = 0.94; p < 0.01). b) faecal loss (r = 0.39; p = 0.29). Each datapoint represents the average for one ewe across the four campaign weeks.

potentially different turnover rates (Streit, 1982), a one-compartment model was used to determine half-lives. Work by Faichney and Boston (1985) demonstrated that the time for equilibration between rumen water and plasma is 3–6 h in sheep. Therefore, a one-compartment model was deemed an appropriate approximation in the current study given the timescale of the experiment.

Despite periods where the ewes likely experienced heat stress (Fig. 2), the half-lives determined in the current experiment are relatively longer than those reported previously in the literature, which are generally between 3 and 7 days (Till and Downes, 1962; Anand and Parker, 1966; Longhurst et al., 1970; Faichney and Boston, 1985), although one study did determine half-lives up to 16 days in some individuals (Anand and Parker, 1966). Since these studies were carried out at least 40 years ago, it is possible that the genetic background of sheep has changed due to selective breeding, resulting in current sheep having greater water use efficiency, and therefore longer body water half-lives. The longer half-lives in the current experiment could also be due to the experimental design. In contrast to the current experiment, in Longhurst et al. (1970), which found the shortest half-lives (2.3 and 3.5 days in summer and winter, respectively), the sheep were fed *ad libitum* and were housed as a group, so they would have moved around more and had the opportunity to engage in other social behaviours and, therefore, had higher metabolic rates. These factors are associated with higher TW intake and faster water turnover (Church, 1993). Moreover, research with cows has shown that their transfer to individual pens for experimental purposes can affect their normal behaviour patterns (Enriquez-Hidalgo et al., 2018). In Till and Downes (1962), however, whose results are similar to those of the current study (half-lives of 3.5–16 days), feed was limited, and the sheep were housed in individual pens, which was also the case in the current study.

3.4. Equilibrium body water isotopic compositions

3.4.1. Comparing water pools

The end-point δ^2H values of blood water, urine water and rumen water are plotted in Fig. 9. As expected, the H isotopic composition of the DW has a strong effect on the water pools (r > 0.99, p < 0.01; Fig. 9a). However, the δ^2H values of the water pools are not the same as that of DW. The water pools are enriched in ^2H relative to DW for the Tap group (by 19‰, 23‰ and 22‰ for urine water, blood water and rumen water, respectively). For the + 250 and + 1000 groups, however, the water pools are depleted in ^2H relative to the respective DW (51–60‰ and 281–342‰ for the +250 and +1000 groups, respectively Fig. 9b).

Urine water is less enriched in ^2H than blood water and rumen water. Potential mechanisms causing isotopic fractionation between the water pools are expected to be independent from DW isotopic composition, thus isotopic relationships between the water pools were investigated by pooling data from all DW groups after normalising for variance (Eq. 5). For the ewes on the ^2H-enriched DW (+250 and +1000 groups), urine water is consistently more depleted than blood water and rumen water, and this is usually the case in the Tap group (Fig. 9c). Blood water is usually more enriched in ^2H than rumen water, but this is not significant (p = 0.06). Wilcoxon Signed Rank tests with a Bonferroni correction (significance level set at 0.017) confirmed that the differences between urine water and blood water and between urine water and rumen water are significant (p < 0.01), but the difference between blood water and rumen water is not significant (p = 0.022)

Water loss through excretion is not generally considered an isotope fractionating route. For example, Schoeller et al. (1986) found no difference in H isotopic composition between blood water and urine water in humans, and this has also been found in birds (McKechnie et al., 2004). However, as in the current study, Abeni et al. (2015) found that urine water was depleted in ^2H relative to blood water in dairy cows, suggesting that the kidneys could be an isotopic fractionation point in ruminants.

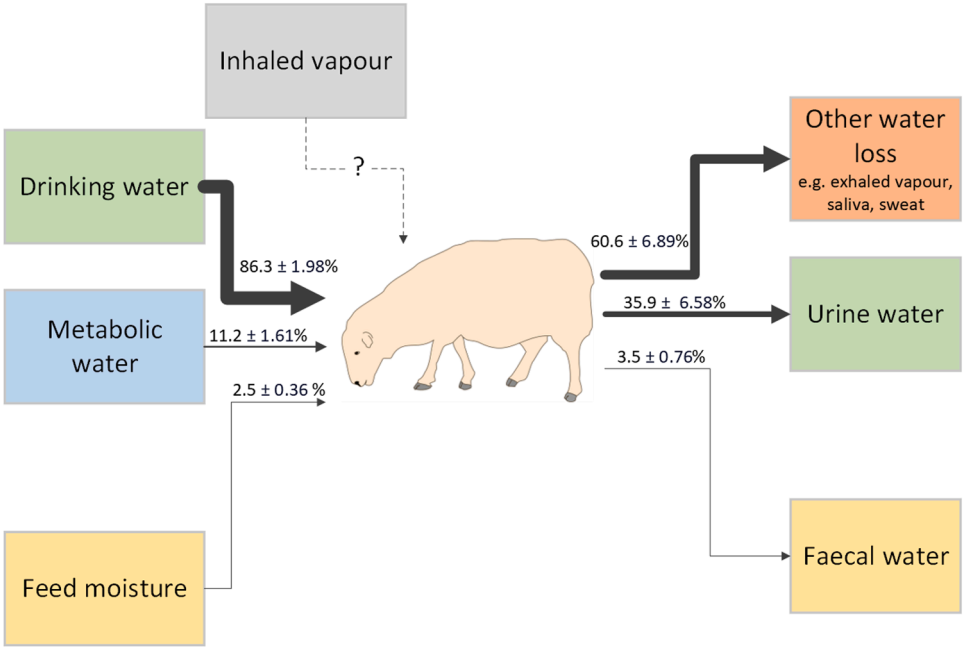

Fig. 6. A model of the water balance in sheep fed at maintenance based on experimental measurements. Green boxes were directly measured during the experiment; yellow boxes were calculated based on experimental measurements; blue boxes were calculated based on literature values; the orange box was calculated to balance the system. The contribution of the grey box (inhaled vapour) was not determined but is likely minimal (Séon et al., 2023). Arrow thickness is proportional to contribution.

3.4.2. *Developing a body water isotope mixing model*

The ^{2}H-enrichment in the water pools relative to DW seen in the Tap group (Fig. 9) can be explained by evaporation (primarily through expiration), which favours the lighter (^{1}H) isotope and therefore results in ^{2}H-enrichment in body water (Schoeller et al., 1986; Wong et al., 1988). This has also been found in other studies in both ruminants (Abeni et al., 2015) and non-ruminants (Wolf and Martinez del Rio, 2000; Wolf et al., 2003; McKechnie et al., 2004). The large ^{2}H-depletion in the water pools relative to the DW in the + 250 and + 1000 groups can partially be explained by the contributions from the other water sources using an isotope mixing model (Eq. 6).

$$\delta^{2}H_{WP} = X_{DW}\delta^{2}H_{DW} + X_{METW}\delta^{2}H_{METW} + X_{FEW} \times \delta^{2}H_{FEW} \quad (6)$$

Where δ^{2}H values are the isotopic compositions and X terms are the fractional contributions of the water sources (drinking water, DW; metabolic water, METW; and feed moisture, FEW) to the water pool (WP).

The δ^{2}H values of feed moisture and metabolic water (METW and FEW terms) were not measured, but they can be estimated. It can be assumed that the δ^{2}H value of the feed moisture is close to that of local meteoric water (-50‰ according to the GNIP database (IAEA, 2024)) and the δ^{2}H value of cellulose, and therefore metabolic water, can be estimated by adding 22‰ to this value (Epstein et al., 1976). The predicted δ^{2}H values of the body water at equilibrium are therefore 207‰ and 985‰ for the + 250 and + 1000 groups, respectively, using Eq. 6. These values are higher (more enriched in ^{2}H) than the measured blood water values by *ca.* 20‰ and 150‰ for the + 250 and + 1000 groups, respectively, indicating that the model requires refining. Nevertheless, these results imply that the contributions of water sources besides DW can reasonably be assumed to account for much of the relative ^{2}H depletion of the water pools compared to the ^{2}H-enriched DW.

This mixing model is an approximation, not least because of the assumptions described above with regards to the H isotopic compositions of metabolic water and feed moisture. The model itself is also an over-simplification since the isotopic fractionation associated with water loss through evaporation, and potentially excretion, is not included. Furthermore, H routing into biomolecules during biosynthesis and H exchange is not captured. Finally, although inhaled water vapour is considered a minor source of water, the H atoms will undergo exchange with body water, thus diluting the ^{2}H signal. A more sophisticated model of H routing, which includes all H sources, pools and sinks, rather than solely water, is therefore an avenue of further work for our group. Additional analyses of tissue H isotopic compositions combined with a box modelling approach using a package such as Isobxr (Tacail, 2023) would provide a more holistic understanding of the routing of the ^{2}H label and enable the development of a correction factor to be applied to body water half-lives determined using isotope tracers.

4. Conclusion

To the authors' knowledge, this is the first time that a long-term DW switch study has been conducted to investigate body water dynamics, and the first time that both urine and blood waters have been analysed to determine half-lives.

The contributions of DW, feed moisture and metabolic water from feed oxidation to TW intake were determined to be 86.3%, 2.5% and 11.2%, respectively. Urine water and faecal water were 90.8% and 9.2% of measured water output. A water balance model demonstrated that measured water output was 39.4% of TW intake, indicating that there is an additional significant route of water loss, likely through expiration, rumination and, to a lesser extent, sweating.

Time-series H isotopic compositions of blood water and urine water demonstrated that they reached isotopic equilibrium, and an exponential model describes ^{2}H-incorporation over time. The half-lives of blood water and urine water were the same and were determined to be *ca.* 16 days in the + 250 group and 10 days in the + 1000 group, with a three-fold variation between the ewes suggesting significant variation in water use efficiency. The difference between the two groups was likely due to higher TW intake in the + 1000 group. These results, using a DW switch approach, yielded similar results to previous studies where a single dose of a tracer is administered, with the longer half-lives in the current experiment likely due to the restricted feed intake experimental design.

The end-point H isotopic compositions of blood water, rumen water

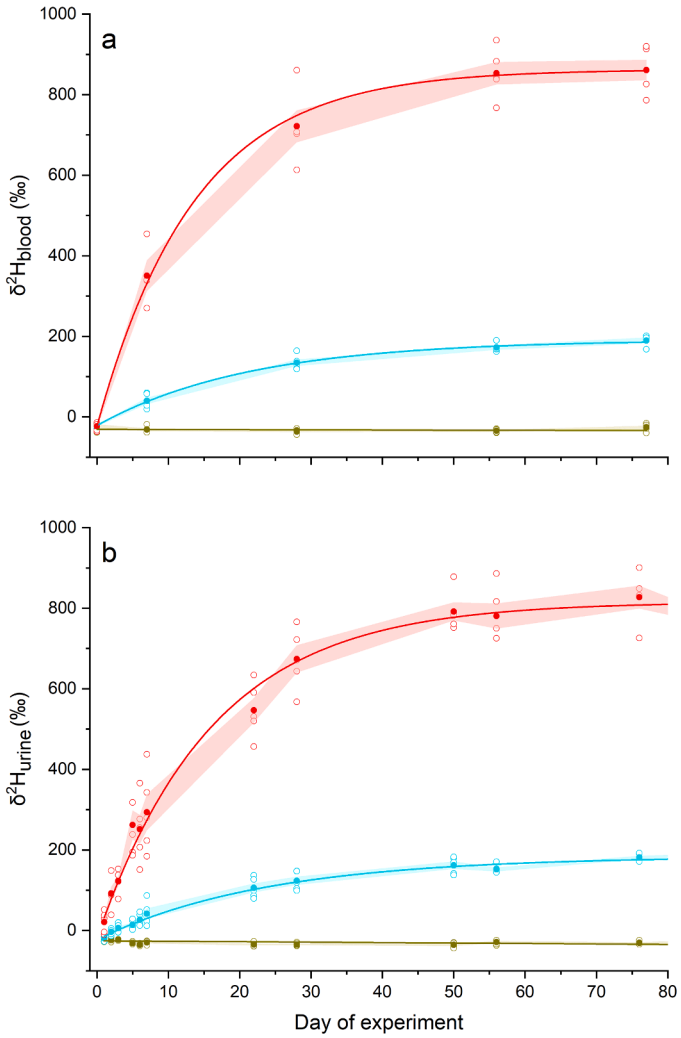

Fig. 7. Time-series plots of the blood water (a) and urine water (b) δ^2H values of the Tap (dark yellow), + 250 (cyan) and + 1000 (red) drinking water groups. Open circles are measured values from individual ewes and filled circles are the mean values at each timepoint within each DW group. Shaded areas represent the standard error of the mean. $R^2 > 0.99$ for the + 250 and + 1000 groups. Values of urine water for one ewe in the + 250 group and blood water for one ewe in the + 250 group could not be obtained.

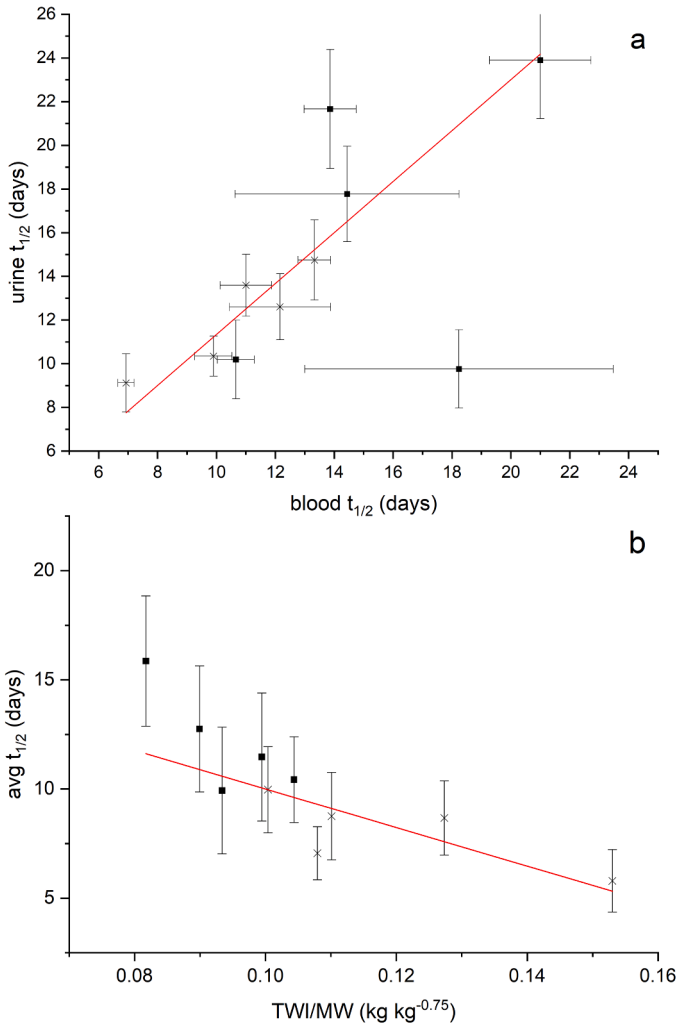

Fig. 8. Body water half-lives. a) Urine water half-live against blood water half-life. Squares and crosses are ewes in the + 250 and + 1000 groups, respectively. Error bars are the propagated error of *a*, used to calculate half-life (Eq. 4). Excluding the outlier, r = 0.9, the slope is 1.2 ± 0.21 ($p < 0.01$) and the intercept is −0.3 ± 2.37. b) Average (blood water and urine water) water pool half-life ($t_{1/2}$) against TW intake per metabolic weight (TWI/MW). Error bars are the propagated error of *a*, used to calculate half-life (Eq. 4). Squares and crosses are ewes in the + 250 and + 1000 DW groups, respectively. r = -0.8; p = 0.01.

Table 4
Determined mean half-lives [standard deviation] of blood water and urine water in the + 250 and + 1000 DW groups. $n = 5$.

Water pool	DW group	Half-life (days)
Blood	+ 250	16 [4.0]
	+ 1000	11 [2.4]
Urine	+ 250	17 [6.5]
	+ 1000	12 [2.3]

and urine water were driven by DW isotopic composition. They were enriched in ^2H relative to DW in the Tap group and depleted relative to DW in the + 250 and + 1000 groups. These results demonstrate that additional factors influence isotopic composition, including evaporation (leading to ^2H-enrichment), contributions from other water sources, particularly metabolic water, and incorporation of water-derived H during tissue biosynthesis. Urine water was depleted in ^2H relative to blood water and rumen water, suggesting that the kidneys are an isotopic fractionation point in sheep.

A mass-balance model was constructed to predict the isotopic composition of body water at equilibrium. This confirmed the assertion that the ^2H-depletion in urine water, blood water and rumen water relative to DW in the + 250 and + 1000 groups was largely because of contributions from other water sources, although there was still a discrepancy between the measured and predicted equilibrium δ^2H values. Including additional factors such as isotope fractionation and H exchange with body tissues is needed to refine the model and improve estimates of water turnover.

This study contributes to the fundamental understanding of water balance and water use efficiency in a temperate sheep breed. The results here demonstrate that water use efficiency is a trait that farmers could select for in temperate sheep breeds to mitigate the impact of climate change and water scarcity on animal productivity. Future work investigating water use efficiency during production (growth, gestation and lactation) and the impact of selection for water use efficiency on productivity and animal health is required to ensure sustainability goals are met.

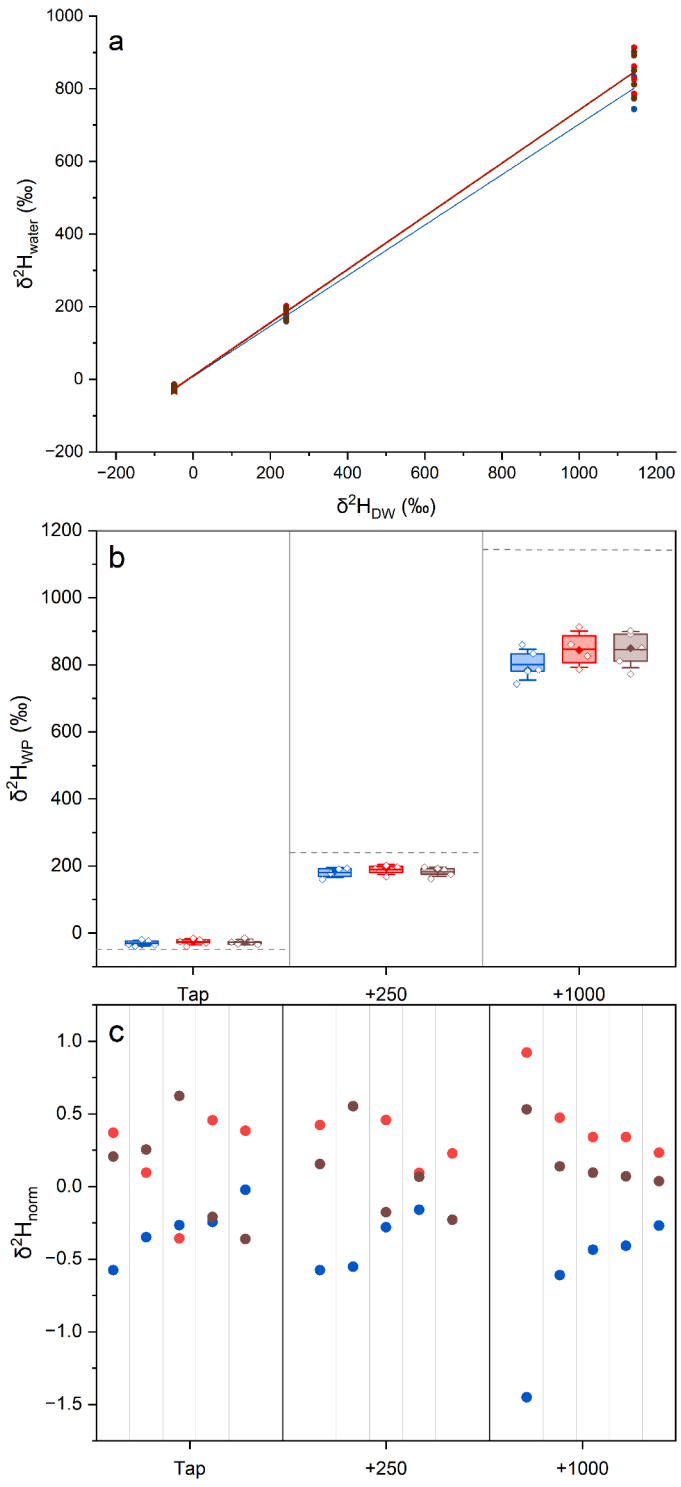

Fig. 9. End-point (Week 12) H isotopic compositions of the water pools (urine water, blue; blood water, red; rumen water, brown). Values of urine water for one ewe in the + 250 group and blood water for one ewe in the + 250 group could not be obtained. a) H isotopic compositions of the water pools (δ^2H_{water}) against isotopic composition of DW (δ^2H_{DW}). $r^2 = 0.99$. b) Box plots of the H isotopic compositions of the water pools for each DW group. Open datapoints are values from individual ewes; filled datapoints are the mean values; and error bars are 1 SD. Dotted lines are the measured δ^2H_{DW} values for each group. c) Normalised δ^2H values.

CRediT authorship contribution statement

Nicky Naylor: Writing – review & editing, Resources, Investigation. **Daniel Enríquez-Hidalgo:** Writing – review & editing, Supervision. **Smith Susanna J. I.:** Writing – original draft, Investigation, Formal analysis, Data curation, Conceptualization. **Fotis Sgouridis:** Writing – review & editing, Resources. **Mélanie Roffet-Salque:** Writing – review & editing, Supervision, Funding acquisition, Conceptualization. **Monica Huerta-Lopez:** Writing – review & editing, Investigation. **Maule Charlie A.:** Writing – review & editing, Methodology, Conceptualization. **Lee Michael R. F.:** Writing – review & editing, Supervision, Conceptualization. **Andrew C. Smith:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Robert G. Wilkinson:** Writing – review & editing, Resources, Conceptualization.

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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