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Isotopic composition of sheep wool records seasonality of climate and diet

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RATIONALE: Hair keratin is a very important material in ecological and archaeological studies because it grows continuously, can be obtained non-invasively, does not require extensive processing prior to analysis and can be found in archaeological sites. Only a few studies have examined seasonal variations in hair isotope values, and there is no published dataset examining the isotope variability recorded in the keratinous tissues of stationary (i.e., non-migrating) domestic mammals.
METHODS: Thirty-six Irish sheep were sampled in eight farms every three months between September 2006 and June 2007. A shearing strategy was adopted to sample only the most recently grown wool in order to represent an average of the summer, autumn, winter and spring conditions. The stable isotope ratios of the ground samples were measured using two different stable isotope mass spectrometers operated in dual-inlet (C, N) and continuous-flow (O, H) mode.
RESULTS: Wool O isotope ratios are a good proxy for seasonal variability in climate and can be used to anchor a chronology independently of other isotope records (C, N) that are influenced by diet or physiology. By contrast, interpretation of seasonal variations in hair H isotope composition in terms of climate is more complex probably due to the influence of dietary H. The C and N isotope values of grass-fed animals varied seasonally, probably reflecting the annual cycle of seasonal variation in grass isotope values. The highest $\delta^{13}C$ values were measured in summer-grown wool, while the highest $\delta^{15}N$ values were measured in winter-grown wool. Supplementation of the sheep diet with concentrates was detected easily and was marked by an increase in $\delta^{13}C$ values and a decrease in $\delta^{15}N$ values in winter-grown wool.
CONCLUSIONS: The present study demonstrates that time-resolved sampling and stable isotope ratio analysis of sheep wool can be used to identify short-term changes in diet and climate and therefore offer a tool to examine a wide variety of present and past husbandry practices.

Since the beginning of the Neolithic about 10,000 years ago until today, humans have exercised an increasing control upon the life cycle of domestic animals.[1] A variety of husbandry strategies have been developed to adapt herding practices when animals are exposed to new environments, or face shortages in water or food supply. While humans can influence many aspects of the life cycle of the animal, the main aspects are reproduction (season and seasonality of birth) and diet (additional supply of food or water, and seasonal movements between different pastures). These management choices can be recorded in the chemical composition of tissues of the animals during their life and can be investigated long after an animal's death. Over the past three decades or so, stable isotope analysis has emerged as a powerful tool to examine different aspects of animal life histories. The stable isotope analysis of light elements such as C, N, S, O and H of biological tissues has been widely used to reconstruct the diet or geographical origin of animals. Stable isotope analysis was first applied to wild fauna in ecological studies.[2,3] It has also been applied to domestic animal products or remains: modern ones for forensic purposes[4–8] as well as ancient ones, to provide insights into different aspects of animal husbandry in the past.[9–11]

When husbandry conditions are radically different (i.e., C_3 pasture *vs* C_4 maize), the analysis of slow integrator tissues such as muscle which turns over continuously can suffice to distinguish between different origins or different farming systems.[4,12] Yet, when herd management differences are more subtle, there is a need to rely on a time-resolved record to take into account the seasonality in herding practices.

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Stable isotope analysis of tooth enamel and dentine has proven useful for reconstructing seasonal changes in the life cycle of wild and domestic animals.[13–18] However, a major limitation of this material is the complexity of the duration and geometry of tooth growth.[19–21] As a result, each sub-sample taken along the tooth axis represents a time-averaged record over 5 to 7 months of growth depending on the tooth or species considered.[22–24] This leads to an attenuation of the environmental variability recorded along the tooth that is inversely proportional to the time of exposure to the new diet.[23–25] Therefore, short-term (daily to weekly) changes between diets of similar isotope values might well go undetected. This is the case, for example, for winter foddering in C_3 environments, where the annual range in plant $\delta^{13}C$ values is only about 2‰.[14,26] Another drawback of teeth is that they cannot usually be sampled while the animal is alive.

Recently, keratinous tissues like hair and hoof have received increased interest as a high-resolution archive of past diets.[27–29] Keratin is a protein that contains the five light elements (C, N, S, O, H) most often used in traceability studies, and samples can be obtained non-invasively and repeatedly, thus facilitating longitudinal studies on the same animal subjects. Because hair grows rapidly and continuously and becomes biologically inactive once formed, stable isotope analysis of hair sections makes it possible to examine fine-scale aspects of the feeding ecology of wild[30–32] and feral mammals.[33] Hair can provide temporally resolved (i.e., sub-weekly) records of animal migration and dietary patterns[31] and of the effect of altitude[34] or environmental changes in the habitat of an animal.[35] Furthermore, keratinous tissues can be preserved over thousands of years under arid or cold climates[36,37] as well as in waterlogged conditions (e.g.[38,39]) and in some mineral environments,[40] and can be used as an alternative to high-crowned teeth in archaeology. Controlled feeding experiments on horses,[27] cattle,[29] and sheep[41] have shown that the C-isotope composition of a new diet is recorded rapidly in hair, making it possible to detect short-term (of the order of days) changes in diet.[42] Thanks to this rapid C turnover, the contribution of previous diets to newly grown hair is minimal, and temporal resolution of dietary history is higher than in any other biological tissue. A quantitative reconstruction of previous diets of animals of different species, age and dietary history can be achieved by a treatment of the isotope data through a multi-pool model.[27,29,43,44] This sampling strategy proved effective in recording plant seasonal variation in $\delta^{13}C$ and $\delta^{15}N$ values over the course of a climatic year.[26]

Most published time-resolved isotope datasets focus on the dietary record (mainly C and N isotopes), and only a few provide an independent record of time. Wool growth rate is affected by several factors that can vary seasonally including photoperiod, pregnancy and parturition, timing of lambing, lactation, nutrition and shearing.[45–47] When animals are reared under controlled conditions, the date of hair collection is known and the isotope sequences can thus be plotted along a time-axis, because tissue growth rates can be assessed independently of the C-isotope values.[27,29,42] This is usually not possible in forensic cases, and virtually impossible in archaeology. Therefore, there is a need to identify an independent marker of time in order to infer possible seasonal changes in diet or location. This is particularly true for domestic animals whose diet can be manipulated and for whom the isotope record and seasonal variations in hair cannot be safely used to infer a chronology.

The H- and O-isotopic composition of environmental water varies widely and systematically across the globe and is incorporated into animal tissues through body water, as well as plant solid matter in the diet, principally carbohydrates, thus offering the possibility of tracing an animal's geographical origin.[48] The O- and H-isotope ratios of meteoric water vary also temporally, with higher values in warm seasons, and lower values in colder season.[49] O and H isotopes are therefore good markers of the seasonality of climate, and can be used to anchor a chronology. Several studies have shown that O and H isotopes can be measured in keratinous tissues.[44,50–56] However, only a few have examined seasonal variations in hair isotope ratios, usually with the goal of tracking large-scale human travel[52,57] and only one[58] examined the variability in O and H isotopes recorded in the keratinous tissues of stationary (i.e. non-migrating) domestic mammals. The O and H in keratin derive from O and H in drinking water, as well as water and organic matter in ingested plants. Literature on seasonal variation in grass cellulose isotope values is scarce but published work on tree-ring cellulose shows that the $\delta^{2}H$ and $\delta^{18}O$ values in cellulose are derived from water and reflect, to a first approximation, precipitation inputs.[59,60] Therefore, seasonal variations in organic H- and O-isotope ratios should follow leaf water signals and ultimately variations in meteoric water isotope composition.

The primary goal of this paper was to examine the pattern of seasonal variations in C, N, O, and H stable isotope ratios in a domestic mammal. Combining these different tracers can potentially provide information on an animal's diet ($\delta^{13}C$ and $\delta^{15}N$ values), nutritional status ($\delta^{15}N$ values), as well as climate ($\delta^{18}O$ and $\delta^{2}H$ values) and this has never been attempted before. We measured the isotopic composition of seasonal wool samples from 36 individual sheep raised in eight different farms across Ireland. Sheep are a good model because, unlike other domestic mammals including cattle and pigs, sheep management is often still extensive and can be (to a degree) used as a proxy for animal management in the past. This study provides a baseline for understanding the magnitude and nature of isotopic variation in modern sheep populations raised under extensive outdoor conditions. A study of the S-isotopic composition of these animals showed that S was a good tracer of distance to the west coast of Ireland and did not change seasonally in stationary animals.[61] For this reason, the S results will not be discussed in detail again. This work also has implication for archaeology since it provides an important means of correctly interpreting the signals measured in incremental tissues commonly found in archaeological sites, such as tooth enamel or dentine.[14,62]

EXPERIMENTAL

Thirty-six sheep were sampled on eight farms (3–5 sheep per farm) in Ireland. The farms were chosen to cover a wide geographical area (Table 1, Fig. 1). All the sheep remained in the same location all year round with the exception of one farm (Donegal) where they were moved 20 km east during winter. On some farms, animals were pasture-fed all year round (hereafter called 'grass-fed'), while on other farms

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Table 1. Location of the farms

County (code in brackets)	Location	Latitude (N)	Longitude (W)	Altitude (asl)
Cavan (CAV)	Ballyhaise	54° 02′	7° 18′	55
Laois (LAO)	Mountrath	53° 01′	7° 33′	165
Donegal (winter) (DON)	Stranorlar	54° 48′	7° 46′	30–35
Donegal (summer) (DON)	Ballinamore	54° 52′	8° 03′	135–240
Galway (GAL)	Athenry	53° 17′	8° 46′	42
Kerry (KER)	Killelane	52° 09′	10° 16′	80
Mayo–lowland (MAY)	Leenane	53° 37′	9° 41′	20
Mayo–hill (MAY)	Leenane	53° 37′	9° 41′	15–275
Meath (MEA)	Ballinabrackey	53° 25′	7° 08′	80
Wicklow (WIC)	Newtownmountkennedy	53° 05′	6° 09′	290

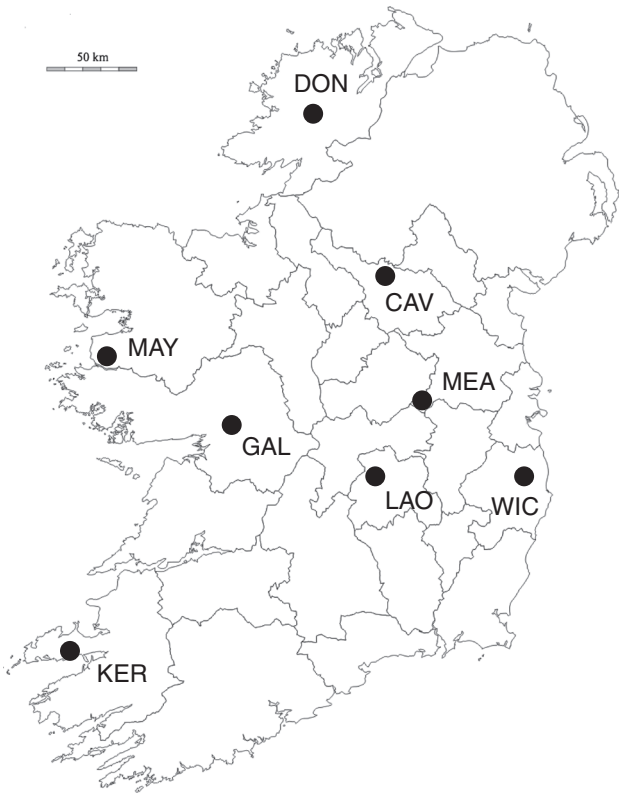

Figure 1. Location of the eight study farms in Ireland. County codes: CAV = Cavan; DON = Donegal; LAO = Laois; GAL = Galway; KER = Kerry; MAY = Mayo; MEA = Meath; WIC = Wicklow. For more details, see Table 1.

the animals were supplemented during the winter months with silage from local grass and/or cereal grain concentrates ('winter supplemented'). Although supplemented, the Donegal flock was considered as 'grass-fed' because it received silage and concentrate during Spring (March and April). Therefore, supplementation did not affect the isotope values of the winter sample, which was taken in March. Detailed information on the dietary history of the animals during the time of the experiment is given in Table 2.

Wool samples were taken every 3 months between September 2006 (sample 1) and June 2007 (sample 4). First, a 15 cm × 15 cm patch of wool was sheared from the side of each animal, using an electric clipper (Oster TURBO A5 single speed clipper, Goodman's, Hzaileah, FL, USA) with a size 10 blade that allowed wool samples to be shorn to 1.2 mm from the skin. This wool was discarded. The actual sample of 1 mm long wool was then sheared to 0.2 mm from the skin using a size 40 blade. This sample was taken at the centre of the patch previously sheared, within a 5 cm × 5 cm square, before cleaning the rest of the square at the same height. This precisely defined, two-step shearing protocol, adopted at each sampling time and applied to the same body location on each animal, was designed to prevent mixing of recently grown wool with 'older' wool and to make wool samples taken at different farms in a given season comparable. Due to tissue turnover, this last millimeter of wool does not just represent the environmental signal during the time of wool growth (approximately 8 to 10 days), but rather an integrated signal over 2–3 months prior to shearing.[41] Therefore, we can assume that the seasonal samples numbered 1 to 4 represent an average of the summer, autumn, winter and spring conditions, respectively.

Sample preparation and isotope analysis

Wool samples were prepared at University College Dublin (Ireland) by sonicating twice for 30 min in warm soapy water, rinsing and oven-drying overnight at 60 °C. The wool was then sonicated twice for 30 min in a mixture of methanol and chloroform (2:1 v/v) to remove lanolin, rinsed with distilled water and oven-dried overnight at 60 °C. The cleaned wool samples were ground to a fine powder using a ball mill and stored in vials until they were weighed for analysis. O and H isotope analysis was carried out at the Stable Isotope Ratio Facility for Environmental Research (University of Utah, Salt Lake City, UT, USA). Ground wool samples were equilibrated for 48 h alongside two keratin laboratory reference materials, and a blind QA/QC (Quality Assurance / Quality Control) keratin material, spanning a wide range of O- and H-isotope ratio values, following previously published methods.[50] Following sample pyrolysis, the H-isotope ratio data were also corrected to account for exchangeable H in keratin-containing materials.[50] The samples and reference materials were loaded (150 μg ±10%) in duplicate in pre-baked 4 × 6 mm silver capsules, equilibrated with a common humidity source, and then desiccated and stored under vacuum at room temperature for at least 72 h prior to analysis to remove adsorbed water. The H- and O-isotope ratios were measured on a Delta Plus XL isotope ratio mass

Table 2. Details of the dietary history of the animals during the study. Sampling dates are also indicated

Animal number	Diet group	2006 Jul	Aug	Sept	Oct	Nov	Dec	2007 Jan	Feb	Mar	Apr	May	Jun
				☆			☆			☆			☆
CAV-1	WS								†				
CAV-2	WS												
CAV-3	WS								†				
CAV-4	WS												
CAV-5	WS												
LAO-1	WS												
LAO-2	WS												
LAO-3	WS												
DON-1	GF	a				b				a		a	
DON-2	GF	a				b				a		a	
DON-3	GF	a				b				a		a	
DON-4	GF	a				b				a		a	
DON-5	GF	a				b				a		a	
GAL-1	WS												
GAL-2	WS												
GAL-3	GF												
KER-1	GF												
KER-2	GF												
KER-3	GF												
KER-4	GF												
KER-5	GF												
MAY-1	WS	c				d	c	indoor				d	
MAY-2	GF	c				d		c				d	
MAY-3	GF	c				d		c				d	
MAY-4	GF	c				d		c					
MAY-5	WS	c				d		c	c			d	
MEA-1	GF												
MEA-2	GF												
MEA-3	GF												
MEA-4	GF												
MEA-5	GF												
WIC-1	WS												
WIC-2	WS												
WIC-3	WS												
WIC-4	WS												
WIC-5	WS												

grass
silage
grass + concentrate
silage + concentrate
GF grass-fed during winter
WS supplementted with concentrate during winter

a : Ballinamore (135-240m asl)
b : Stranorlar (30m asl)
c : hill (15-275m asl)
d : lowland (15m asl)

☆ sampling date
† deceased

spectrometer (ThermoFinnigan, Bremen, Germany) operated in continuous-flow mode. The samples were pyrolysed at 1400 °C in a high-temperature conversion elemental analyzer (TC/EA), producing H_2 and CO gas. Solid samples were introduced into the pyrolysis column via a zero blank autosampler (Costech Analytical, Valencia, CA, USA) and the gases were separated on a 1-m, 0.25 in (o.d.) molecular sieve 5 Å gas chromatography column (Costech Analytical).

For C- and N-isotope analysis, 700 µg ± 10% were weighed into 4 × 6 mm pressed tin capsules. The working standard was 1 mg leucine, prepared by freeze-drying 50 mL of a 20 mg mL^{-1} stock solution into tin cups. Two working standards were run after every ten samples, the first used for quality control, and the second as the reference and for drift correction. The working standards had previously been calibrated against 'Europa flour' and IAEA standards N1 and N2. Additional leucine and flour standards were included at the end of each run to check run-to-run consistency. The C- and N-isotope ratios were measured at Iso-Analytical Ltd (Crewe, UK) using a ANCA–NT 20-20 stable isotope analyser with an ANCA–NT solid-liquid preparation module (Europa Scientific Ltd, Crewe, UK) operated in dual mode to measure both C- and N-isotope ratios in one sample.

The stable isotope values are reported using the standard delta notation (δ per mil, ‰) calculated as follows: $\delta X\ (‰) = [(R_{sample} - R_{reference}) - 1]$, where X is the element considered, and R is the ratio of the heavy to light stable isotope (e.g., $^{13}C/^{12}C$) in the sample (R_{sample}) and the standard ($R_{reference}$). The results are reported against international standards: VSMOW ($\delta^{18}O$ and $\delta^{2}H$ values) VPDB ($\delta^{13}C$ values), and AIR ($\delta^{15}N$ values). The analytical precision (at 1σ) achieved for the standards that were analysed along with the samples was 0.1‰ for both the C- and the N-isotopic values, and 1.5‰ and 0.2‰ for the H- and O-isotopic values, respectively.

Statistical analysis

The stable isotope ratios measured for each element and season were summarized as mean and standard deviation values. Statistical analysis was carried out using the Past 2.03 software package.[63] Shapiro-Wilk tests for normality showed that the distributions of the $\delta^{13}C$, $\delta^{15}N$ and $\delta^{2}H$ values were normal, but that of the $\delta^{18}O$ values was not ($p < 0.05$). Therefore, non-parametric statistical tests were used throughout the study.

RESULTS

Data reporting

The stable isotope values measured on seasonal samples are provided in Supplementary Table S1 (see Supporting Information) and summary statistics are given in Table 3. Seasonal variations in stable isotope values are plotted for

Table 3. Summary statistics of wool stable isotope ratios measured on individual Irish sheep

				$\delta^{13}C$ (‰)					$\delta^{15}N$ (‰)					$\delta^{2}H$ (‰)					$\delta^{18}O$ (‰)				
County	#[a]	diet group	n[b]	min	max	range	Mean	SD	min	max	Range	Mean	SD	min	max	range	mean	SD	min	max	range	Mean	SD
Cavan	1	WS	2	-27.0	-26.3	0.7	-26.6	0.5	9.3	10.0	0.8	9.7	0.5	-93.9	-92.7	1.2	-93.3	0.8	12.7	15.0	2.2	13.8	1.6
Cavan	2	WS	4	-26.8	-24.2	2.5	-25.8	1.1	7.6	10.8	3.2	9.6	1.4	-99.0	-91.7	7.3	-95.0	3.3	14.0	15.9	2.0	15.0	0.8
Cavan	3	WS	2	-26.7	-26.4	0.4	-26.5	0.3	9.4	11.7	2.3	10.6	1.6	-91.4	-88.5	2.9	-89.9	2.0	14.3	15.4	1.1	14.8	0.8
Cavan	4	WS	4	-26.9	-24.9	2.0	-26.1	0.9	7.8	11.9	4.1	9.6	1.7	-102.5	-88.4	14.2	-92.4	6.8	14.2	16.9	2.7	15.5	1.1
Cavan	5	WS	4	-27.1	-24.2	2.9	-25.9	1.3	7.5	12.1	4.6	9.8	1.9	-107.5	-94.0	13.5	-97.9	6.5	13.5	14.9	1.5	13.9	0.7
Donegal	1	WS	4	-27.8	-25.4	2.4	-26.8	1.0	3.0	11.6	8.6	8.0	3.7	-102.9	-88.0	14.9	-95.3	8.1	13.3	14.8	1.5	14.1	0.7
Donegal	2	WS	4	-27.2	-25.1	2.1	-26.3	1.0	2.7	11.6	8.9	7.4	3.9	-98.8	-86.1	12.6	-94.0	5.9	13.3	15.4	2.1	14.2	1.0
Donegal	3	WS	4	-27.1	-24.7	2.4	-26.2	1.1	2.7	11.4	8.7	7.4	4.0	-103.0	-89.7	13.3	-97.7	5.8	12.7	15.1	2.4	14.1	1.1
Donegal	4	GF	4	-27.4	-25.6	1.7	-26.7	0.7	6.8	11.5	4.8	9.0	2.2	-102.3	-90.5	11.8	-96.3	4.9	12.9	15.2	2.2	14.0	1.2
Donegal	5	GF	4	-27.4	-25.1	2.3	-26.5	1.0	2.2	10.8	8.6	7.4	3.7	-102.6	-90.6	12.0	-98.1	5.5	13.1	15.9	2.8	14.3	1.2
Galway	1	GF	4	-27.5	-25.9	1.6	-26.6	0.7	6.9	9.3	2.4	8.1	1.0	-94.8	-85.8	9.0	-90.9	3.7	12.3	16.7	4.3	14.4	2.2
Galway	2	GF	4	-27.7	-25.5	2.3	-26.6	1.0	6.5	9.4	2.9	7.7	1.3	-97.9	-85.2	12.7	-92.4	5.3	12.5	17.3	4.7	15.2	2.2
Galway	3	GF	4	-28.0	-26.2	1.8	-27.3	0.8	8.0	8.9	0.8	8.4	0.4	-102.2	-86.4	15.8	-96.3	6.9	11.9	16.4	4.5	14.5	2.1
Kerry	1	WS	4	-27.6	-25.9	1.8	-26.6	0.7	7.9	9.6	1.7	8.8	0.9	-102.4	-89.9	12.6	-95.7	5.9	13.2	15.6	2.4	14.5	1.3
Kerry	2	WS	4	-27.6	-25.9	1.8	-26.6	0.7	7.6	9.7	2.1	8.8	1.0	-93.2	-85.6	7.7	-91.0	3.7	13.6	16.8	3.2	15.3	1.5
Kerry	3	GF	4	-27.6	-26.2	1.4	-26.8	0.6	6.9	10.0	3.0	8.8	1.3	-95.5	-87.0	8.5	-91.4	4.5	13.4	16.4	2.9	15.1	1.4
Kerry	4	GF	4	-27.5	-26.2	1.3	-26.8	0.5	7.8	9.7	1.9	8.9	0.9	-95.7	-89.5	6.1	-91.9	2.7	13.8	16.7	2.8	15.4	1.2
Kerry	5	GF	4	-27.6	-25.9	1.7	-26.8	0.7	7.2	10.1	3.0	8.8	1.4	-102.7	-87.8	14.9	-93.7	6.8	14.1	16.5	2.5	15.2	1.1
Laois	1	GF	4	-26.4	-24.9	1.6	-25.9	0.7	6.8	7.8	1.1	7.3	0.5	-99.5	-85.9	13.6	-94.5	5.9	12.1	16.4	4.4	14.3	2.2
Laois	2	GF	4	-25.9	-25.0	0.9	-25.5	0.4	6.5	7.8	1.3	7.2	0.6	-102.1	-90.8	11.3	-95.9	4.7	12.3	16.7	4.4	14.3	2.1
Laois	3	GF	4	-25.9	-25.0	0.9	-25.6	0.4	6.2	7.3	1.2	6.9	0.5	-99.0	-92.4	6.6	-96.2	2.8	12.1	16.4	4.3	14.1	2.2
Mayo	1	WS	4	-26.8	-24.4	2.4	-25.5	1.1	3.2	8.4	5.2	5.8	2.6	-89.3	-82.7	6.6	-86.2	3.5	13.3	18.1	4.8	15.6	2.3
Mayo	2	GF	4[c]	-26.5	-24.6	1.9	-25.8	0.8	3.4	6.6	3.2	5.5	1.4	-90.7	-86.8	3.9	-88.4	2.1	12.9	16.9	4.1	14.5	2.2
Mayo	3	GF	4	-26.4	-24.4	2.0	-25.6	0.9	3.0	5.7	2.7	4.5	1.2	-96.5	-82.6	14.0	-91.9	6.3	12.7	18.0	5.3	15.2	2.7
Mayo	4	GF	4	-26.8	-24.5	2.3	-25.7	1.0	2.8	6.6	3.8	5.2	1.7	-94.8	-86.4	8.4	-89.9	3.6	13.7	17.7	4.0	15.6	1.8
Mayo	5	WS	4	-26.7	-24.1	2.5	-25.6	1.1	3.1	6.8	3.7	5.5	1.6	-100.8	-82.0	18.8	-90.0	9.3	13.8	16.9	3.1	15.4	1.3
Meath	1	GF	3	-28.9	-26.3	2.7	-27.2	1.5	5.9	8.7	2.7	7.0	1.4	-108.4	-97.5	10.9	-102.8	5.5	12.6	16.0	3.4	14.6	1.8
Meath	2	GF	4	-28.0	-25.4	2.6	-26.6	1.1	5.0	6.7	1.7	5.8	0.8	-103.0	-87.8	15.2	-96.7	6.4	11.8	16.1	4.3	13.6	1.9
Meath	3	GF	4	-28.0	-25.6	2.5	-26.6	1.1	5.3	7.8	2.5	6.4	1.1	-99.1	-87.2	12.0	-92.2	5.3	12.3	17.3	5.1	14.5	2.3
Meath	4	GF	4	-28.0	-25.2	2.8	-26.5	1.2	6.0	7.9	1.9	6.8	0.8	-99.5	-89.3	10.2	-94.1	4.6	12.5	16.6	4.1	14.2	2.0
Meath	5	GF	4	-28.1	-26.2	1.9	-26.8	0.9	4.9	7.2	2.2	6.2	1.0	-106.8	-92.8	13.9	-99.4	5.7	12.0	15.6	3.5	13.7	1.6
Wicklow	1	WS	4	-26.3	-26.0	0.4	-26.1	0.2	5.6	7.7	2.0	6.5	0.9	-103.7	-90.3	13.3	-96.8	5.5	13.0	15.7	2.7	14.3	1.2
Wicklow	2	WS	4	-27.3	-25.2	2.1	-26.1	0.9	5.7	8.4	2.6	6.9	1.2	-92.6	-83.4	9.2	-88.2	5.0	12.8	16.5	3.7	14.8	1.8
Wicklow	3	WS	4	-27.2	-26.1	1.0	-26.5	0.5	5.5	7.1	1.7	6.2	0.8	-95.2	-86.3	8.9	-90.7	4.4	12.4	16.5	4.1	14.9	1.8
Wicklow	4	WS	4	-27.3	-25.4	1.9	-26.4	0.8	5.8	8.0	2.2	6.9	1.2	-100.4	-89.2	11.2	-93.4	4.9	13.9	16.6	2.7	15.4	1.3
Wicklow	5	WS	3	-27.1	-26.0	1.1	-26.5	0.6	5.4	7.2	1.8	6.6	1.0	-99.5	-93.1	6.4	-95.5	3.5	12.6	14.3	1.7	13.6	0.9

[a]animal number in each farm.
[b]number of samples taken from each individual. Some animals did not provide four samples because they either died during the experiment (Cavan), could not be found on the day of sampling (Wicklow) or because the analysis failed (Mayo).
[c]n = 3 for O and H.

all individuals together (Fig. 2) and for grass-fed (Fig. 3) and winter-supplemented sheep separately (Fig. 4). Seasonal variations in the stable isotope values for individual sheep are plotted in Supplementary Figs. S1 to S8 (see Supporting Information).

Carbon-isotope ratios

The wool C-isotope ratios varied between –28.9‰ and –24.1‰, showing an annual variability of 4.8‰ at the country scale. The most positive values were measured for summer-grown wool (–25.5 ± 0.7‰), while the most ^{13}C-depleted values were measured for autumn- and winter-grown wool (–26.7 ± 0.5‰). For each sampling season, the inter-individual variability was between 2.2‰ and 2.3‰, except for the winter-grown wool where it was twice as high (4.7‰). Winter wool from grass-fed sheep showed significantly (Mann Whitney U test, $p < 0.001$) lower δ^{13}C values (average: –27.4 ± 0.8‰; n = 21) than wool from sheep supplemented with concentrates (average: –25.6 ± 0.7‰; n = 13). Overall, the intra-individual variability (i.e., the amplitude of seasonal variation measured for each individual) in the δ^{13}C value was between 0.3 and 2.8‰; it was higher but less variable for grass-fed sheep (2.1 ± 0.4‰) than for supplemented sheep (1.5 ± 0.8‰).

Nitrogen-isotope ratios

The wool N-isotope ratios varied between 2.2‰ and 12.1‰, showing an annual variability of 10.0‰ at the country scale. The most ^{15}N-enriched values were measured for autumn- and winter-grown wool, while the most ^{15}N-depleted values were measured for summer- and spring-grown wool. For each sampling season, the inter-individual variability was between 6.1‰ and 7.6‰. The seasonal pattern was more clearly visible in the grass-fed sheep than in the supplemented sheep. In grass-fed sheep, the wool δ^{15}N values increased steadily from summer (5.3 ± 2.3‰) to winter (8.8 ± 2.0‰). The summer wool δ^{15}N value was significantly lower than that for the autumn and winter wool (Mann-Whitney U test, $p < 0.001$) in grass-fed but not in supplemented sheep. Supplementation resulted in a marked decrease in the average wool δ^{15}N value between autumn (8.9 ± 2.0‰) and winter (7.6 ± 0.6‰). The seasonal variability in δ^{15}N values was more prominent in grass-fed sheep (between 0.8 and 8.9‰) than in supplemented sheep (between 0.8 and 4.6‰). On average, the intra-individual variability was higher for grass-fed sheep (3.9 ± 2.6‰) than for supplemented sheep (2.3 ± 1.1‰). Among grass-fed sheep, the highest seasonal variability was found in the western farms (Mayo and Donegal) due to the low values (between +2 and +4‰) measured in summer-grown wool.

Oxygen-isotope ratios

The wool O-isotope ratios varied between 11.8‰ and 18.1‰, suggesting an annual variability of 6.3‰ at the country scale. The most ^{18}O-enriched values were measured for summer- and spring-grown wool (15.8 ± 0.8‰ and 16.1 ± 1.0‰, respectively), while the most ^{18}O-depleted values were measured for autumn- and winter-grown wool (13.1 ± 0.8‰ and 13.6 ± 0.9‰, respectively). A lower inter-individual variability in δ^{18}O values was found for summer (2.8‰) than for the other three growing seasons (ranging between 3.4 and 4.3‰). No differences in δ^{18}O values were found between winter wool from grass-fed and winter-supplemented sheep. Overall, the intra-individual variability in the δ^{18}O value was between 1.1 and 5.3‰. The average intra-individual

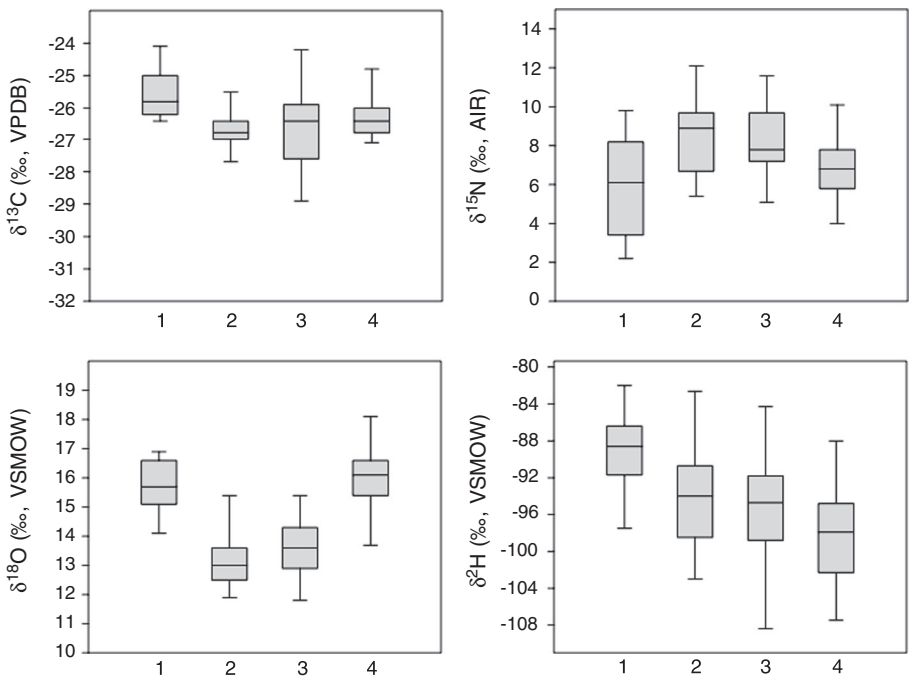

Figure 2. Boxplot of the seasonal variation in wool stable isotope ratio values across all sites and for all animals. Seasons: 1 summer; 2 autumn; 3 winter; 4 spring. Each boxplot is based on data from eight sites, with 3–5 animals per site.

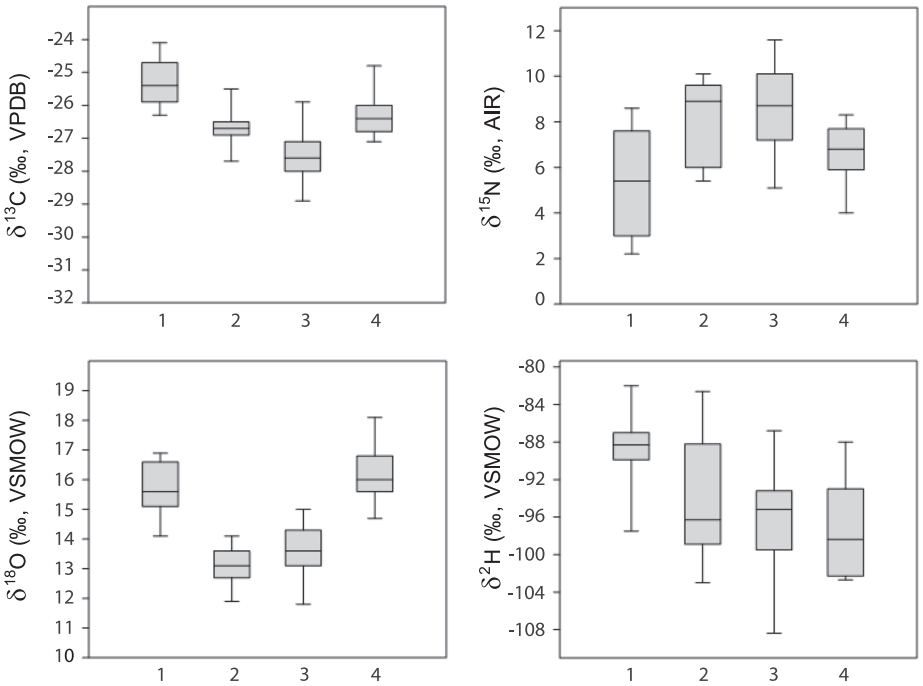

Figure 3. Boxplot of the seasonal variation in wool stable isotope ratio values across all sites, for grass-fed animals only. Seasons: 1 summer; 2 autumn; 3 winter; 4 spring. Each boxplot is based on data from five sites (GAL, KER, MAY, MEA, DON) with five animals per site except GAL (n = 1).

variability was 3.3 ± 1.1‰ and this was not significantly different for grass-fed and for supplemented sheep (Mann-Whitney U test, $p = 0.50$).

Hydrogen-isotope ratios

The wool H-isotope ratios varied between –108‰ and -82‰, showing an annual variability of 26‰ at the country scale. The most ^{2}H-enriched values were measured for summer wool (–89.1 ± 3.7‰), while more ^{2}H-depleted, but similar δ^{2}H values were measured for autumn- (–94.2 ± 5.3‰), winter- (–95.0 ± 5.5‰) and spring-grown wool (–97.4 ± 4.8‰). The summer wool δ^{2}H values were significantly different from those for the autumn, winter and spring wool (Mann-Whitney U test, $p < 0.001$). A lower inter-individual variability in δ^{2}H values was found for summer (16‰) than for the other three growing seasons (ranging between 20 and 24‰). When each season was treated separately, no difference was found between grass-fed and winter-supplemented sheep. Overall, the intra-individual seasonal variability in δ^{2}H values was between 1.2 and 18.8‰. The average intra-individual variability was slightly higher for grass-fed (11.6 ± 3.7‰) than for supplemented sheep (9.4 ± 4.0‰), but this difference was not significantly different (Mann-Whitney U test, $p = 0.15$). Once the results had been pooled, the average intra-individual variability was 10.7 ± 3.9‰.

DISCUSSION

Our results show that the stable isotope ratios of wool record significant short-term variations in diet and climate, thus demonstrating the relevance and capability of our time-resolved sampling strategy. In the discussion below, we will first examine the record of climatic variability in animal hair O-isotopic values, and then move to the dietary record (C- and N-isotopic values), which is more directly under the herder's control. Finally, we will discuss the H-isotope record, which appears to be under the combined influence of climate and diet.

The O-isotope record of climatic seasonality in wool

The wool O-isotope ratios displayed a clear seasonal signal, with the most ^{18}O-enriched values measured in the summer samples, and the most ^{18}O-depleted in the winter samples. The pattern of variation observed in wool closely tracks the seasonal pattern of meteoric water δ^{18}O in mid-high latitudes of the northern hemisphere,[64] and we suggest that it records seasonal fluctuation in local environmental δ^{18}O values. This result is consistent with a model proposed to account for the O-isotope record of a water switch in rodent hair.[44] According to this model, the δ^{18}O value in hair is controlled by the isotopic composition of the gut water which itself is related to the O-isotopic composition of body water and food. A mass balance model calculation showed that drinking water was responsible for 56% of the O in body water, and 45% of the O in hair; the reaction progress variable approach suggested that turnover of O in rodent hair was rapid, and that 83% of the hair O had turned over in 13 days.[44] If this estimation is also valid for sheep, about half of the seasonal variability in the O isotope of environmental water should be recorded in hair. Sheep can obtain their water directly, from drinking, or indirectly, through water ingested in plants. The precipitation isotope composition was not measured, but the values can be predicted for the different farm locations

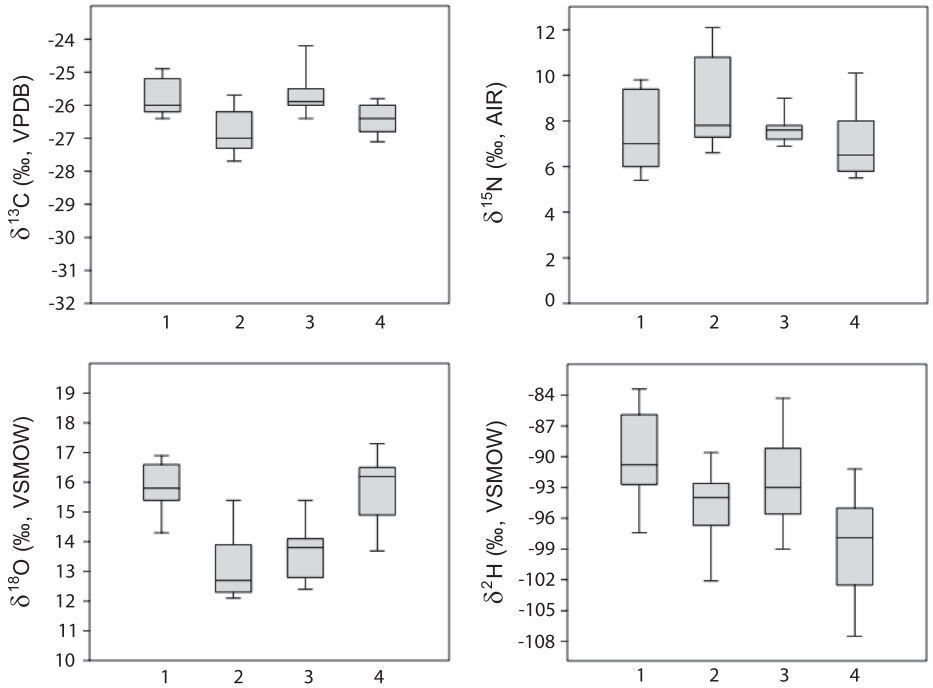

Figure 4. Boxplot of the seasonal variation in wool stable isotope ratio values across all sites, for winter-supplemented animals only. Seasons: 1 summer; 2 autumn; 3 winter; 4 spring. Each boxplot is based on data from four sites (CAV, LAO, GAL, WIC) with 2–5 animals per site.

using the Online Isotope Precipitation Calculator[65] (OIPC). The results from using the OIPC suggest that, on average, the annual range of variation in precipitation O-isotopic values in Ireland is 4‰. If animals were to obtain their water directly from meteoric water, we would expect a seasonal variability in the $\delta^{18}O$ values of wool of slightly less than 2‰. The average intra-individual seasonal variability in wool was higher (3.3 ± 1.1‰) and only four of the 36 animals showed a seasonal variability of less than 2‰ (Table 3). This result suggests that animals do not obtain their water directly from meteoric water, but from evaporated sources (at least during summer). It is likely that the $\delta^{18}O$ value of drinking water, which is usually obtained from tap water, will be less influenced than that of plant water by seasonal variations in temperature and humidity. Informal discussion with the farmers indicated that unlike cattle, grass-fed sheep do not need much additional water as they find most of the water they need in the plants they eat. The situation is different for supplemented animals that are fed dry food (mainly concentrate) and tend to drink more water to meet their water-balance requirements. Animals selected in our study were only supplemented during the winter time (Table 2), a period where plant $\delta^{18}O$ values are not significantly affected by evaporation, and we suggest that this could explain the lack of difference observed between the hair $\delta^{18}O$ values of grass-fed and winter-supplemented animals. All the animals selected for the study were fed grass outdoors during the summer, a period where evapo-transpiration is highest (even in Ireland!). This could also explain why the average $\delta^{18}O$ values in the two feeding groups were not significantly different during summer.

The C- and N-isotope record of diet in grass-fed sheep

The C- and N-isotope ratio values of grass-fed sheep exhibited a clear seasonal signal (Fig. 3). The highest $\delta^{13}C$ values were measured during summer, while the lowest values were measured during winter. The opposite pattern was observed for N, with higher $\delta^{15}N$ values during winter and lower values during summer. As a result, the C- and N-isotopic values were negatively correlated ($r^2 = 0.56$, $p < 0.01$) (Fig. 5). The pattern observed for C isotopes mimics the seasonal variation observed in the grass $\delta^{13}C$ values, probably due to seasonal variation in water availability.[66] Seasonal samples of grass were not systematically taken in all farms, but a recent study on pasture-fed cattle demonstrated that 75–90% of seasonal variations in grass $\delta^{13}C$ values were picked up in hair.[26] The average seasonal variation measured by Osorio *et al.*[26] for cattle raised in Ireland was similar to that measured here for grass-fed sheep (about 2‰). The turnover of C in sheep wool is rapid[41] and, because the rate of change in grass $\delta^{13}C$ values is relatively slow and continuous, we propose that wool captures most of the amplitude of grass isotope variations.

We observed that, on average, sheep wool C-isotope values were 1.5‰ more positive than for cattle tail hair in the study by Osorio *et al.*[26] It is noteworthy that the grass-fed cattle in that study and the grass-fed sheep from County Meath come from the same region but still showed this difference; so the difference in location does not explain this case. Previous studies have suggested that the diet–hair shift in $\delta^{13}C$ values is similar in sheep and cattle, at close to 3‰.[26,34,41] Thus, the difference that we observed is rather explained by the fact that cattle and sheep do not feed from the same type of

Rapid Communications in Mass Spectrometry

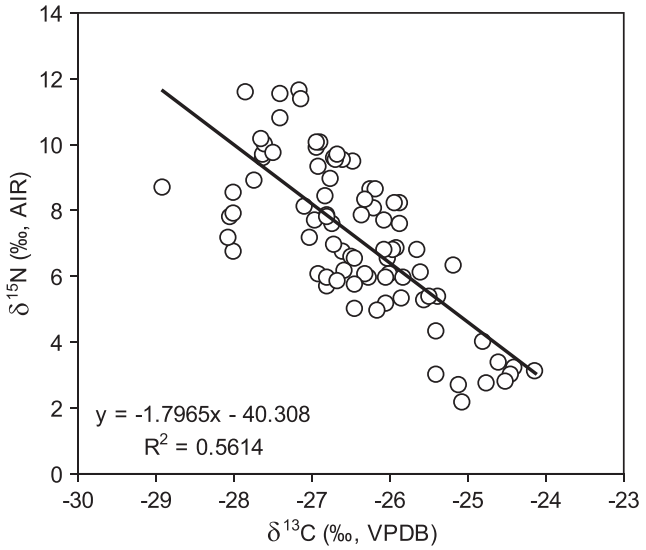

Figure 5. Correlation between C- and N-isotope ratios in wool from grass-fed sheep (all sites and seasons pooled, n = 83, $p < 0.01$).

vegetation. A comparative study showed that sheep and cattle grazing together have different diets because sheep can graze deeper within the sward canopy and have a greater ability to select from fine-scale mixtures.[67] As a result, sheep tend to select much more herbs than cattle, ingest more dead/senescent plant parts, and avoid tall grass flower stems. This difference in grass selection could explain the difference in wool $\delta^{13}C$ values since different parts of the same plant can display some isotope variability, with green leaves having $\delta^{13}C$ values 1–2‰ lower than stems or senescent leaves.[68]

All the grass-fed animals exhibited a similar pattern of a seasonal variability in wool $\delta^{15}N$, but large differences in amplitude were observed between the different locations. The largest seasonal shifts were observed in the Donegal and Mayo sheep (Supplementary Figs. S2 and S6, see Supporting Information). In these farms, a large intra-individual variability up to 10‰ could be measured. These animals were moved between different pastures over the course of the year, spending summer on hilly pasture and winter on lowland pastures (Table 2). Given the magnitude of this shift, it is probably related to a change in the isotopic composition of the diet. The variability in plant N-isotopic values depends on different factors such as soil conditions, N fertilization (mineral and organic), N availability, different pathways of N assimilation, N recycling within a plant, climate, altitude and distance from sea.[34,69] Taking into account the diet-wool shift in N-isotope ratios of 3‰ proposed for sheep[34] and the average $\delta^{15}N$ values of 3‰ for winter wool in Mayo and Donegal, we can estimate that grass $\delta^{15}N$ values in the hill farms were close to 0‰. This is similar to what Maennel *et al.*[34] measured in Alpine pastures at low- to mid-altitude. This contrasts with the $\delta^{15}N$ value of about 6 ± 2‰ calculated for lowland pasture based on the average summer wool $\delta^{15}N$ value of grass-fed sheep. This $\delta^{15}N$ value is similar to the value measured by Osorio *et al.*,[26] and we propose that a difference in soil condition and pasture management intensity between the hills (unimproved pastures) and the lowland (improved pastures) as well as the type of vegetation is responsible for the difference measured between the summer and winter wool of these animals.

It is interesting to note that wool from grass-fed stationary animals (animals that did not move between different fields) also showed an increase in $\delta^{15}N$ values (although less pronounced) during the autumn and winter months. For these animals (such as in Kerry or Meath), the intra-individual variability was between 1.7 and 3.0‰ (Table 3, and Supplementary Figs. S4 and S7, see Supporting Information). Similar patterns in $\delta^{15}N$ increases in animal tissues during winter have also been observed for both wild and domestic animals.[14,31,36] These authors usually interpret this shift as reflecting a dietary change in relation to a change in habitat/location. For example, Makarewicz[14] measured a 0.8 to 2.2‰ increase in $\delta^{15}N$ along serially sampled tooth collagen of the second molar of modern caprines from Mongolia, which was interpreted by the author as the result of a movement to a manured pasture during winter. No clear correlation was observed between C- and N-isotopic values, but this could be due to the ingestion of some C_4 plants by the animals. A 4‰ winter increase in $\delta^{15}N$ values was also recorded in the hair of African elephants, and interpreted as a change in diet (and location) at that time.[31] Finally, a 2.6‰ winter increase was found in the hair of Pleistocene mammoth from the Arctic.[36] Again, the authors explained this trend as a dietary stress during winter, forcing the mastodont to incorporate greater amounts of woody plants and/or mosses in its diet. We cannot invoke animal movement to explain the results obtained on the grass-fed stationary sheep in the present study. The most straightforward explanation for the variations that we observed is a seasonal change in the isotopic composition of grass. A recent survey carried out in Scotland showed that ryegrass C- and N-isotope values anticovaried seasonally.[68] Green leaves or stems of ryegrass showed a 3 to 4‰ annual variability in their N-isotope ratios, with the highest values measured during autumn and winter, and the lowest values measured during spring and summer. This seasonal pattern was explained by the application of mineral fertilizers (characterized by low $\delta^{15}N$ values, around 0‰) during spring and the plant uptake of N deriving from mineralization of organic N during autumn and winter. Other factors such as the quality of the diet,[70] or poor physiological condition,[71] can also contribute to modify wool or hair $\delta^{15}N$ values. These factors were not controlled for, and it is therefore difficult to argue for one factor rather than another. Because intra-individual variations up to 3‰ can be measured in stationary animals raised on the same diet, caution is required when using N isotopes as a mobility tracer.

The C- and N-isotope record of diet in winter-supplemented sheep

Winter-supplemented animals deviated from the general seasonal pattern described above and were detected easily. Winter supplementation caused an increase in wool $\delta^{13}C$ values (up to 2.9‰) and a decrease in wool $\delta^{15}N$ values (up to 4.6‰) (Supplementary Fig. S1, see Supporting Information). This blurred the seasonal pattern observed in grass-fed animals as shown by the absence of a correlation between C- and N-isotopic values in supplemented sheep

($r^2 = 0.09$). The isotope effect appeared very clearly in animals that were supplemented 2–3 months prior to sampling (Cavan, Laois, Wicklow). In Galway, animals 1 and 2 were fed silage plus concentrate and showed a 2‰ change in their wool $\delta^{13}C$ and $\delta^{15}N$ values between autumn and winter (Supplementary Fig. S3, see Supporting Information). Animal #3 was fed silage only and its isotope values remained constant, suggesting that concentrates rather than silage were responsible for this shift. Concentrates can vary in composition, but often contain various amounts of maize,[72] thus explaining the increase in winter wool $\delta^{13}C$ values. The pattern observed in sheep wool $\delta^{15}N$ values was similar to that observed in the hair of cattle that were fed pasture grass followed by concentrate in sequence.[26] In the latter experiment, the hair $\delta^{15}N$ values of cattle fed concentrate were on average 3–4‰ lower than those of animals fed grass outdoors. This broadly corresponds to the $\delta^{15}N$ difference measured in the foodstuffs and was explained by the authors as being caused by the presence of legumes (soybean) in the concentrate. Legumes can fix N_2 directly from air, leading to lower N-isotope ratios than grass that mostly assimilates soil inorganic N as ammonium or nitrate.[73] Systematic measurement of grass, straw and concentrate isotope values for each of the studied farms was beyond the scope of this project. However, soybean (and maize) are two key elements in concentrates provided to sheep and it is likely that their presence contributed to the observed decrease in $\delta^{15}N$ values during winter.

Grass growing in Ireland and Britain is characterized by more negative $\delta^{13}C$ values than those in the rest of Europe, and this difference is recorded in the isotopic composition of the animal tissues.[26,72] From a forensic perspective, supplementation with C_3 plants grown outside NW Europe can be detected easily. C-isotope values in animal tissues have been used as a tracer of winter foddering with seaweed or C_4 plants in modern and ancient caprines.[14,74] Winter foddering with local C_3 plants might be more difficult to identify. We suggest that intra-individual variability lower than 2‰ could be interpreted as a sign of winter foddering, but the local input signal needs to be established, ideally by stable isotope analysis of local grass sampled on a monthly basis. Nowadays, supplementation is usually provided to support ewes during pregnancy. We can thus expect that this would affect females rather than males and could contribute to increased inter-individual variability at the flock scale.

Hydrogen isotopes in wool: a tracer of climate and diet

As with O, the H-isotope ratios of meteoric water vary temporally, with higher values in warm seasons, and lower values in colder season.[49] While we expected H-isotope ratios to be a good marker of the seasonality of climate in wool, our results indicated that the seasonal pattern of the H-isotope signal was not as clear as for O. Only in one location (Meath; Supplementary Fig. S7, see Supporting Information) did wool H-isotope ratios exhibit a clear seasonal pattern with high values during summer and lower values during winter, although usually the signal was less predictable, even within a single flock (Supplementary Figs. S1–S6 and S8, see Supporting Information). As a result, correlation between H and O was poor when all seasons were plotted together ($r^2 = 0.11$) (Fig. 6). The correlations

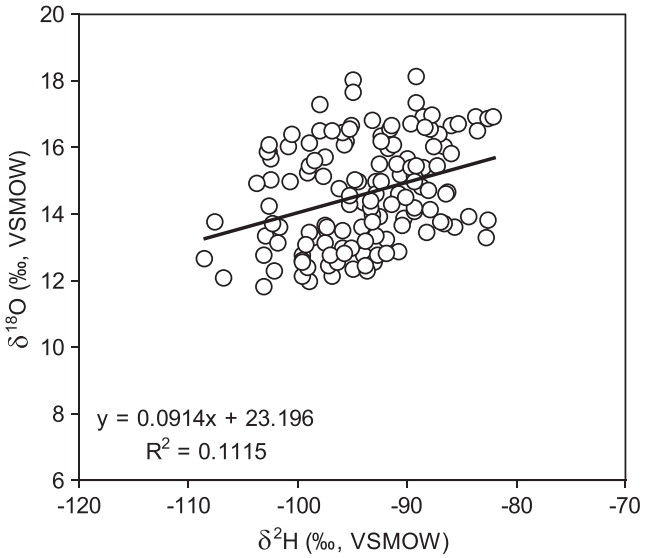

Figure 6. Correlation between O- and H-isotope ratios in Irish wool (all sites and seasons pooled, n = 137, $p < 0.01$).

slightly increased but remained weak when the seasons were plotted separately ($r^2 = 0.26$, 0.22, 0.40 and 0.35 for summer, autumn, winter and spring, respectively). Our results are in contrast with those obtained by Kirsanow *et al.*,[75] who showed that variations in H-isotope ratios in the tooth dentine of modern caprines from Mongolia followed a clear seasonal pattern. They are, however, in keeping with O'Brien and Wooller[57] who found a poor correlation ($r^2 = 0.18$) between O- and H-isotope ratios in facial hair of a human subject travelling between two areas with distinct water isotope values. In continental environments such as Mongolia, seasonal variations in meteoric water δ^2H values are high (170‰) and this probably explains why, although attenuated, a seasonal pattern can still be easily detected in animal organic tissues. In more temperate environments like Ireland, seasonal variations in precipitation δ^2H values are much smaller (about 25‰ according to the OIPC) and the influence of H derived from other sources might become preponderant.

Water-switching experiments on humans,[53] a rodent,[44] and quail[76] indicated that drinking water accounts for a smaller portion of the H-isotope signal in hair for the O-isotope ratios. For instance, Podlesak *et al.*[44] found that while drinking water was responsible for 71% of the H in the body water of a small rodent, it only accounted for 25% of the H in hair. This result is in agreement with a previous estimate for feathers (26–32%) and nails (27%) from quail.[76] For humans, the data presented by O'Brien and Wooller[57] (their Fig. 4) allow us to estimate that, while 67% of the O in human hair is derived from drinking water, only 29% of the H in human hair derived from this pool. This is in line with the estimate of 27% proposed by Ehleringer *et al.*[52] for human hair. If these experimental results also apply to sheep, only about 30% of H found in hair is derived from body water. The contribution of environmental water H to sheep wool H (25%) is about half of that of environmental water O to hair O (45%), and this is probably why the seasonal signal in hair H is not as clear as for O. The remainder is derived from H chemically bound in food constituents such

as amino acids, carbohydrates and fat. Therefore, it is likely that the pattern of seasonal variability in hair δ^2H values is blurred by the contribution of the different sources of H from food provided to the animals.

By looking at the data for each flock separately, it appears that winter supplementation with concentrate has an effect on animal δ^2H values. For example, the Wicklow sheep recorded a 3 to 8‰ increase in their δ^2H values following consumption of concentrates during winter (Supplementary Fig. S8, see Supporting Information). In Galway, the winter wool δ^2H values were 10‰ higher for the two winter-supplemented sheep than for the third one which was grass-fed (Supplementary Fig. S3, see Supporting Information). The lack of significant difference between winter wool from grass-fed and supplemented sheep at the country scale may be explained by the confounding effects of geography and diet. First, the 10 to 15‰ eastward decrease across Ireland in meteoric and surface water δ^2H values due to progressive distillation of the air masses as rain tracks west to east[77] probably has an impact on the δ^2H value of the local grass, and therefore of the wool. Secondly, farmers may use different concentrate brands, containing cereals that are usually not produced locally, and this could also contribute to blurring of the isotope signal in the winter wool at the regional scale. However, the trends detected in several farms suggest that H isotopes could become a new marker of winter supplementation in domestic animals in addition to C or N isotopes. Additional work is needed to better understand seasonal (and inter-annual) variations in plant H-isotope ratios as well as the factors governing dietary H incorporation in keratin before we can use wool δ^2H values as a tracer of seasonal change in diet.

CONCLUSIONS

This pilot study demonstrates that the isotope values of Irish sheep wool exhibit considerable seasonal variability. This variability is governed by environmental and climatic factors, but also by husbandry practices. Domesticated animals record during their lives several short-term changes in diet that can be induced naturally (by the environment) or anthropogenically (by the herder). It is important to keep in mind that each of the isotope tracers measured in wool keratin can be influenced by different types of constraints. Although further work is required, our approach using multiple isotope systems proved useful in differentiating between environmentally, anthropogenically or metabolically driven short-term changes in diet and physiology and therefore provides a tool to examine a wide variety of husbandry practices in the present but also in the past. Wool contains a wealth of information on herding practices, and our sequential sampling strategy could be applied to other continuously growing keratinous tissues such as horn or hooves.[28,78] Application to other tissues such as tooth dentine which is more commonly found in archaeological sites is also possible, but will require adapting the sampling strategy to the geometry of tissue growth, or at least to account for this bias by analyzing the $\delta^{18}O$ values of tooth dentine together with other isotopes related to the animal diet.

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