Do grazer hair and faeces reflect the carbon isotope composition of semi-arid C3/C4 grassland?

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Abstract

The carbon isotope composition ($\delta^{13}C$) of C3/C4 mixed grassland is reflected in the $\delta^{13}C$ of diet, hair or faeces of grazers, if ^{13}C discrimination ($^{13}\Delta$) between grassland vegetation and these tissues is known and constant. However, these relationships could be modified by selective grazing or differential digestibility of the C3 and C4 components, potentially creating a bias between grassland and grazer tissue $\delta^{13}C$. Importantly, these factors have never been studied in detail. We investigated the relation between $\delta^{13}C$ of C3/C4 grassland vegetation and that of faeces and hair of sheep in a 3-year (2005–2007) experiment in the Inner Mongolian semi-arid steppe. The experiment employed six stocking rates (0.375–2.25 sheep ha^{-1} year^{-1}; four replications), which allowed for a large variation in species composition, digestibility, and diet selection. Faecal-nitrogen content, a proxy for digestibility, decreased from 1.9% to 1.5% during the grazing period due to aging of the herbage. At the same time, the C3/C4 ratio decreased due to the later growth initiation of C4 species. $^{13}\Delta$ between diet and faeces ($^{13}\Delta_{DF}$; 0.6‰) and between diet and hair ($^{13}\Delta_{DH}$; −3.9‰) were not influenced by stocking rate, period in the season or C3/C4 ratio. Moreover, faeces–hair discrimination ($^{13}\Delta_{FH}$; −4.3‰), which reflects differences between digestibility of the C3 and C4 components, did not vary along the different gradients. The $\delta^{13}C$ of grassland vegetation can be estimated from the $\delta^{13}C$ of sheep faeces and hair, provided that $^{13}\Delta$ was accounted for. This is useful for landscape- or regional-scale investigations or reconstruction of C3/C4 vegetation distribution from faeces and hair, which provide different temporal and spatial integration of grassland isotope signals.
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Zusammenfassung

Die Kohlenstoff-Isotopenzusammensetzung ($\delta^{13}C$) von C3/C4 Grasland widerspiegelt sich im $\delta^{13}C$ des Futters, der Haare sowie des Kots von Weidetieren, sofern die ^{13}C-Diskriminierung ($^{13}\Delta$) zwischen der Vegetation und dem jeweiligen Tierparameter bekannt und konstant ist. Unterschiedliche Verdaulichkeiten der C3 und C4 Komponenten,

oder selektiver Fraß können jedoch $^{13}\Delta$ verändern und somit zu Fehlern bei der Schätzung des $\delta^{13}C$ von Grasland führen. Diese Zusammenhänge wurden bisher noch nicht systematisch untersucht. In der vorliegenden Studie untersuchten wir deshalb die Beziehung zwischen dem $\delta^{13}C$ von gemischtem C3/C4 Grasland und dem Kot und den Haaren von Schafen. Die Studie wurde in einem mehrjährigen Beweidungsexperiment (2005–2007) mit sechs verschiedenen Besatzdichten (0.375 bis 2.25 Schafe pro ha, vier Wiederholungen) in der semi-ariden Steppe der Inneren Mongolei durchgeführt. Dieses Versuchsdesign ermöglichte eine große Variation der Artenzusammensetzung, der Verdaulichkeit und der Futterselektion. Der Kotstickstoffgehalt (ein Maß für die Verdaulichkeit) sank während der Weideperiode von 1.9% auf 1.5%. Dieser Effekt beruhte auf der Alterung des Pflanzenbestandes. Gleichzeitig nahm das C3/C4-Verhältnis durch den späteren Austrieb der C4 Arten ab. Die $^{13}\Delta$ zwischen Futter und Kot ($^{13}\Delta_{DF}$; 0.6‰) und zwischen Futter und Haar ($^{13}\Delta_{DH}$; −3.9‰) war unabhängig von der Besatzdichte, dem Zeitpunkt innerhalb der Weideperiode und dem C3/C4-Verhältnis. Des Weiteren wurde die Kot-Haar Diskriminierung ($^{13}\Delta_{FH}$; −4.3‰) – ein Maß für Verdaulichkeitsunterschiede zwischen C3 und C4 Arten – ebenfalls nicht von den verschiedenen Gradienten beeinflusst. Diese Untersuchungen zeigen, dass das $\delta^{13}C$ von C3/C4 Grasland in der Inneren Mongolei aus dem $\delta^{13}C$ von Kot oder Haaren bestimmt werden kann, sofern die (konstante) $^{13}\Delta$ berücksichtigt wird. Diese Ergebnisse sind besonders für lokale und regionale Untersuchungen, bzw. die Rekonstruktionen des C3/C4-Verhältnisses nützlich, da Kot und Haare Isotopensignale über (unterschiedlich lange) räumlich-zeitliche Skalen integrieren.

Introduction

The carbon isotope composition of a primary consumer (herbivore) is a reflection of the photosynthetic mechanism of the primary producers on which it subsists (DeNiro & Epstein 1978). This relationship between feed and animal isotope composition is of great value for the reconstruction of C3 and C4 vegetation patterns, since C3 and C4 plants differ strongly in carbon isotope composition ($\delta^{13}C$) (O'Leary 1981). This isotope signal is propagated in the food web, which is based on C3 or C4 vegetation (e.g. Tieszen, Boutton, Tesdahl, & Slade 1983; Sponheimer et al. 2006; Barbosa, Köhler, Auerswald, Lüps, & Schnyder 2009). In consequence, fossil animal tissues (teeth and bone tissue) can be used as surrogates for plant remains, which may not be available. In the same way, animal tissues can also be used as an isotopic archive of contemporaneous vegetation composition and change. This is especially valuable in the case of grassland vegetation since this consists of short-lived plants. In particular, it is interesting for monitoring changes in the proportions of C3 and C4 plants in mixed grasslands.

As animals move across landscapes they feed on spatially and temporally distributed resources, ingesting isotopic information over space and time. Hair appears to be particularly useful for recording (and integrating) such isotopic information, because it is durable, chemically uniform, and grows at an approximately constant rate over an extended period of time (Schwertl, Auerswald, & Schnyder 2003; Ayliffe et al. 2004; Schnyder, Schwertl, Auerswald, & Schäufele 2006). Faeces, if collected at intervals, provide better resolved temporal records of animals' diets (Codron, Lee-Thorp, Sponheimer, de Ruiter, & Codron 2006; Jones & Hu 2006), with the additional advantage that animals need not be captured for sample collection.

The isotopic reconstruction of grassland vegetation from animal tissue or faeces isotope composition relies on the assumptions that there is a known constant relationship between the $\delta^{13}C$ of vegetation and that of the tissue or faeces. In the case of C3/C4 grassland this requires that (1) animals do not prefer one photosynthetic type over the other (non-selective feeding) and (2) the C3 and C4 components have similar digestibility, so that they are assimilated and excreted in similar proportions. These are important assumptions, which need testing because presently the relationships between diet, tissue and faeces are extrapolated from only a handful of experiments of mostly domestic species, and have small sample sizes. This is especially true for C3/C4 grassland systems such as the grasslands of Central Asia, the largest contiguous biome of the world, which is grazed extensively by domestic animals, particularly sheep, goats and cattle. However, even these species are thought to graze selectively, choosing a diet of better quality than the average vegetation (Prache, Roguet, & Petit 1998). This could create a significant bias between hair $\delta^{13}C$ ($\delta^{13}C_{hair}$) or faeces $\delta^{13}C$ ($\delta^{13}C_{faeces}$) and that of vegetation, as C4 plants are believed to be less nutritious and less digestible than C3 plants (Caswell, Reed, Stephens, & Werner 1973; Barbehenn, Chen, Karowe, & Spickard 2004). We are not aware of any systematic investigation of the relationship between the $\delta^{13}C$ of natural C3/C4 grassland vegetation and that of hair or faeces of bulk grazers such as sheep. In particular, it is unclear if differential selection and digestibility of C3 and C4 vegetation components affect this relationship.

Keratinous tissue (such as hair) is generally enriched in ^{13}C relative to diet by approximately 3‰ in domestic and wild ruminants fed pure C3 or C4 diets (Minson, Ludlow, & Troughton 1975; Auerswald et al. 2009). Conversely, faeces are commonly slightly depleted (−0.6‰) relative to diet ($\delta^{13}C_{diet}$; Jones, Ludlow, Troughton, & Blunt 1979; Coates, Van der Weide, & Keer 1991; Norman, Wilmot, Thomas, Masters, & Revell 2009). These isotopic deviations are termed discrimination ($^{13}\Delta$) and are attributed to isotope fractionation during digestion or metabolism (e.g. DeNiro & Epstein 1978; Tieszen et al. 1983). They are calculated as

$$^{13}\Delta = \frac{\delta^{13}C_{diet} - \delta^{13}C_{tissue}}{1 + \delta^{13}C_{tissue}} \qquad (1)$$

with $\delta^{13}C_{tissue}$ denoting either $\delta^{13}C_{hair}$ for estimation of diet–hair discrimination ($^{13}\Delta_{DH}$) or $\delta^{13}C_{faeces}$ for estimation of diet–faeces discrimination ($^{13}\Delta_{DF}$).

These discriminations are often treated as constants (e.g. Sponheimer et al. 2003; Codron et al. 2007; Norman et al. 2009) although there is some variation in the data. Certainly, some (and perhaps most) of the variation is due to error, such as sampling and analytical error or isotopic non-equilibrium between diet and tissue when slowly turning over body pools contribute to hair growth and defecation (Ayliffe et al. 2004; Zazzo, Moloney, Monahan, Scrimgeour & Schmidt 2008; Auerswald et al. 2009). While the variation in discrimination may be of little relevance, if C3 diets are compared with C4 diets due to the large isotopic spacing between both, the spacing becomes small and the variation in discrimination becomes relevant if the objective is to detect a change in diet from C3/C4 from 0.8/0.2 to 0.7/0.3. In such cases it is important to know, under which conditions, discrimination is constant and when it changes as a function of animal, diet, habitat or environment.

Where ruminants graze freely in C3/C4 grassland, the isotopic composition of the herbage eaten (diet) is different from the isotopic composition of the vegetation (= herbage on offer) as it is influenced by diet selection, which is usually unknown. However, the relationship between vegetation ^{13}C and hair ^{13}C (i.e. vegetation–hair discrimination, $^{13}\Delta_{VH}$) provides a clue about selection given that digestibility is identical and isotopic scrambling in the rumen distributes C3- and C4-derived components evenly among microbial protein: $^{13}\Delta_{VH}$ should be < −3‰, if animals prefer the C3 component, and > −3‰ if the C4 component is preferred. In the same way, vegetation–faeces discrimination ($^{13}\Delta_{VF}$) should be < 0.6‰, if animals prefer C3, and > 0.6‰ if C4 is preferred. Furthermore, the comparison of vegetation–hair and vegetation–faeces discrimination should provide a clue about the relative digestibilities of the C3 and C4 components of the diet. If the ingested C4 is less digestible than the C3, then the C4 will contribute a larger fraction of the total faecal output, causing a less positive diet–faeces discrimination. Consequently, the C4 contribution to hair growth would be smaller, hence causing a less negative diet–hair discrimination and both causing a two-fold effect on the faeces–hair discrimination. Therefore, selection and digestibility have different effects on diet–hair and diet–faeces discrimination, and can thus be distinguished based on ^{13}C analysis of vegetation (herbage on offer), hair and faeces. Whereas selection would influence $^{13}\Delta_{DF}$ and $^{13}\Delta_{DH}$ in the same direction, effects of differential digestibility should have opposite effects (Fig. 1). As far as we know, these putative effects have never been explored with domestic ruminants grazing C3/C4 grassland.

This work was conducted to shed light on this issue, and in particular to explore the potential of sheep hair and faeces as archives of the carbon isotope composition of Inner Mongolia C3/C4 grassland. The study was
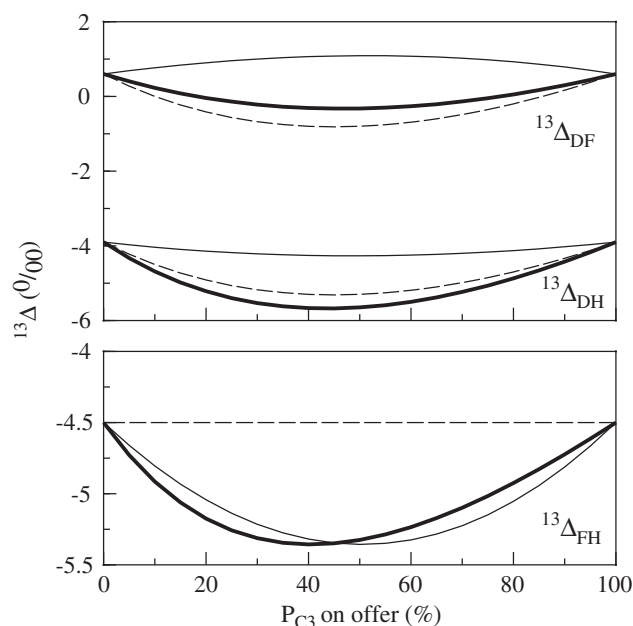
Fig. 1. Modeled apparent diet–tissue discriminations (top box: diet–faeces discrimination, $^{13}\Delta_{DF}$, and diet–hair discrimination, $^{13}\Delta_{DH}$) and resulting faeces–hair discrimination, $^{13}\Delta_{FH}$ (bottom box) with increasing relative abundance of C3 plants on offer. In the calculations, we assumed a higher digestibility of the C3 component and a higher selection of the presumably more palatable C3 component: solid lines denote a better digestibility of the C3 component (D3 = 60% and D4 = 54%) without selection (S3 = S4 = 100%); the dashed lines denote a preference of the C3 component over the C4 component (S3 = 120% and S4 = 80%) at the same digestibility (D3 = D4 = 56%); the bold lines denote the combined effect of higher digestibility and higher selection of the C3 component (D3 = 60%, D4 = 54%, S3 = 120% and S4 = 80%). Other input parameters were taken as constants (values that are applicable to the research area; see Eqs. (4)–(11): d$\delta^{13}C$ = −12.1‰, p$^{13}\Delta_{DF}$ = 0.6‰, p$^{13}\Delta_{DH}$ = −3.9‰).

conducted in the frame of an extensive grazing experiment, with a wide range of stocking rates which determined opportunities for selective grazing of C3 and C4 components. There was little opportunity for selection at high stocking rates (= high grazing pressure), but ample opportunity for selection at low stocking rates (= low grazing pressure). Effects of selection and digestibility were analyzed in terms of diet–hair and diet–faeces discrimination (as predicted in Fig. 1).

Material and methods

Grazing experiment

The grazing experiment was situated at 116°40′E and 43°33′N in the Xilin River Basin, Inner Mongolia Autonomous Region, People's Republic of China. It was operated by the Inner Mongolian Grassland Ecosystem Research Station (IMGERS), the Institute of Botany, Chinese Academy of Sciences, and the Deutsche Forschungsgemeinschaft research group 536 MAGIM (www.magim.net). The vegetation is a semi-arid steppe, dominated by perennial C3 grasses (mainly *Stipa grandis* and *Leymus chinensis*), but perennial C4 grasses (mainly *Cleistogenes squarrosa*) and annual C4 herbs (mainly *Salsola collina*) were also present

The growing period in Inner Mongolia lasts from April/May to September/October. During this period the mean nitrogen content of aboveground biomass decreases from about 2.1–0.7% (Liu 1993). Growth of C4 plants starts about 30–50 days later (Bai, Han, Wu, Chen, & Li 2004).

The grazing experiment consisted of six different stocking rates (fixed stocking rates of 0.375 to 2.25 sheep ha^{-1} year^{-1}, in steps of 0.375 sheep ha^{-1} year^{-1}). The sheep were about 2 years old non-pregnant and non-lactating ewes from the local Mongolian fat-tail breed.

Vegetation samples were taken at around 1st to 16th of July, August or September of 2005, 2006 and 2007. Bulk vegetation samples were obtained from outside of three frames of 0.5 m^2 in pasture cages (hereafter cage) and from the inside of pastures (hereafter pasture). Thus, we determined the proportion of vegetation ingested by the animals. In both situations, vegetation was clipped at 1 cm stubble height. After clipping, the cages were moved to a previously grazed part to determine growth for the subsequent month. In total 217 vegetation samples from cages and 178 vegetation samples from pastures were analyzed. At the same time, fresh faeces were sampled in 2005 and in 2006. In 2007, faeces sampling was performed at around 20th to 30th of June, July and August. In total, we analyzed 152 faeces samples. In addition, in 2007, we sampled the leaves of the dominant C3 species (*S. grandis*, $n = 72$) and C4 species (*C. squarrosa*, $n = 72$) in the grazed area.

Hair samples originated from shearing at the end of the grazing period in late September of the years 2005, 2006 and 2007. This included only hair grown during the grazing period because as sheep had also been shorn just before the beginning of the grazing period (June). In total, 40 hair samples were analyzed.

Sample preparation, isotope and elemental analysis

Plant and faeces samples were dried prior to milling: plant samples for 48 h at 60 °C and faeces for 48 h at 40 °C. Plant and faeces samples were ground to homogeneity with a ball mill; 0.7–0.8 mg of plant or faeces material were packed into tin cups for isotope analysis. The distal first cm of the hair samples was discarded. This accounted for the stubbles left after shearing, which were marked by dye, to account for the proportion of hair within the hair channel and turnover of body pools. The remaining hair (approximately 4–6 cm) was cleaned by the procedure of Schwertl et al. (2003), ground to homogeneity with a ball mill, and 0.2–0.4 mg hair material was packed into tin cups for isotope analysis.

The $\delta^{13}C$ and the nitrogen content of each sample were determined with an elemental analyzer (NA 1110; Carlo Erba, Milan) interfaced (ConFlo III; Finnigan MAT, Bremen) to an isotope ratio mass spectrometer (Delta Plus; Finnigan MAT). Carbon isotopic data are presented as $\delta^{13}C$, with $\delta^{13}C = (R_{sample}/R_{standard})-1$, where R is the $^{13}C/^{12}C$ ratio and standard is the Vienna Pee Dee Belemnite standard. Each sample was measured against a laboratory working standard CO_2 gas, which was previously calibrated against an IAEA secondary standard (IAEA-CH6, accuracy of calibration 0.06‰ SD). After every tenth sample a solid internal lab standard (SILS) with similar C/N ratio as the sample material (fine ground wheat flour for plant and faeces material, and protein powder for hair material) was run as a blind control. The SILS were previously calibrated against an international standard (IAEA-CH6). The precision for sample repeats was better than 0.15‰ (standard deviation).

All data on $\delta^{13}C$ of vegetation, faeces and wool and the data on nitrogen content of vegetation and faeces are available in Tables 1–3 in Appendix A.

Calculation of dietary $\delta^{13}C$ and ^{13}C discrimination

The $\delta^{13}C$ of the diet ($\delta^{13}C_{diet}$) was estimated using an isotopic mass balance:

$$\delta^{13}C_{diet} = \frac{\delta^{13}C_{cage} \times DM_{cage} - \delta^{13}C_{pasture} \times DM_{pasture}}{DM_{cage} - DM_{pasture}}$$

(2)

with $\delta^{13}C$ of aboveground biomass samples taken inside the cage ($\delta^{13}C_{cage}$) and on the pasture ($\delta^{13}C_{pasture}$) and

the aboveground dry biomass of the respective samples taken inside the cage (DM_{cage}) and on the pasture ($DM_{pasture}$). The estimated $\delta^{13}C_{diet}$ may have a large error when subtrahend and minuend in the denominator are similar. This was not the case as DM_{cage} was on average 38% higher than $DM_{pasture}$.

The different ^{13}C discriminations (diet–hair, diet–faeces, faeces–hair) could then be calculated from the measured isotopic composition of the different tissues and the dietary ^{13}C according to Eq. (1). Additionally, the proportion of C3 plants (P_{C3}) and C4 plants ($P_{C4} = 1 - P_{C3}$) at each site was estimated from the isotopic composition of the aboveground biomass following Auerswald et al. (2009).

Faecal-nitrogen content, digestibility and relative feed intake

Faecal-nitrogen content ($\%N_{faeces}$) can be used as a proxy for digestibility (Boval, Archimede, Fleury, & Xande 2003; Wang et al. 2009). This relation is based on the decreasing amount of diet and increasing relative amount of undigested ruminal microbial protein excretion in faeces as dietary digestibility increases. Mean digestibility of organic matter was estimated using the equation by Wang et al. (2009), which has been derived in the same environment. Relative feed intake was calculated as

$$\text{relative feed intake} = \frac{DM_{cage} - DM_{pasture}}{DM_{cage}} \qquad (3)$$

Statistical methods and outliers

Linear regressions and multiple non-linear regressions (weighted least-squares estimations) were used to evaluate the datasets. The coefficients of determination were tested with a two-sided test for significance of the regressions. Regression coefficients were tested on parity with Student's t-test. Hypothesis testing on equal means of groups or on parity of the mean of the population and a specified value were carried out using Student's t-test (two-sided). This was generally performed against a 95% confidence interval, preceded by a test for normal distribution and if necessary by an F-test on parity of variances. Mean values are presented with 95% confidence intervals (CI) prefixed by a plus-minus sign. Significance thresholds of $P \geq 0.05$ (n.s.), $P < 0.05$ (*), $P < 0.01$ (**) and $P < 0.001$ (***) were used. All procedures followed standard protocols (Sachs & Hedderich 2006) and were carried out using GNU R 2.9.0 (R Development Core Team 2009). Two $\delta^{13}C$ outliers in the vegetation data and seven $\%N_{faeces}$ outliers were removed from the data set.

Modeling of apparent diet–tissue and tissue–tissue discriminations

The apparent diet–tissue and tissue–tissue discriminations were modeled with the following equations:

$$S4 = 2 - S3 \qquad (4)$$

$$sC4 = \frac{S4 \times (1 - P_{C3})}{S4 \times (1 - P_{C3}) + S3 \times (P_{C3})} \qquad (5)$$

$$sC3 = 1 - sC4 \qquad (6)$$

$$dC4 = \frac{D4 \times sC4}{D4 \times sC4 + D3 \times sC3} \qquad (7)$$

$$rC4 = \frac{(1 - D4) \times sC4}{(1 - D4) \times sC4 + (1 - D3) \times sC3} \qquad (8)$$

$$^{13}\Delta_{DF} = p^{13}\Delta_{DF}(1 - P_{C3} - rC4) \times d\delta^{13}C \qquad (9)$$

$$^{13}\Delta_{DH} = p^{13}\Delta_{DH}(1 - P_{C3} - dC4) \times d\delta^{13}C \qquad (10)$$

$$\frac{^{13}\Delta_{FH} = 1 + {}^{13}\Delta_{DF}}{1 + {}^{13}\Delta_{DH}} - 1 \qquad (11)$$

where S4 denotes the relative selectivity of C4 biomass, S3 the relative selectivity of C3 biomass, P_{C3} the relative contribution of C3 species to aboveground biomass, sC3 the selected C3 biomass, sC4 the selected C4 biomass, dC4 the digested proportion of C4 biomass, D4 the digestibility of the ingested C4 biomass, D3 the digestibility of the ingested C3 biomass, rC4 the residual proportion of C4 biomass in the faeces, $p^{13}\Delta_{DF}$ the 'pure' diet–faeces discrimination, $p^{13}\Delta_{DH}$ the 'pure' diet–hair discrimination, $d\delta^{13}C$ the difference of $\delta^{13}C$ between C3 and C4 species, and $^{13}\Delta_{FH}$ the faeces–hair discrimination. Here, digestibility is defined as the relative fraction of the either C3- or C4-derived biomass in faeces to the ingested respective biomass. Relative selectivity is defined as the ratio of ingested C3- or C4-derived mass to the respective mass in the vegetation and 'pure' discrimination as the $^{13}\Delta$ arising for an infinite feeding of one component (i.e. not influenced of either the digestibility or selectivity).

Results

Seasonal change in abundance and leaf nitrogen content of C3 and C4 plants

P_{C3} on offer, calculated from $\delta^{13}C$ of bulk vegetation from the cages, decreased significantly during the growing period ($P \ll 0.001$; not shown) due to the later growth initiation of C4 plants (Bai et al. 2004). On the 170th day of the year (mid of June) approximately 95% of aboveground biomass consisted of C3 plants, while

Table 1. Mean differences between the carbon isotopic composition of vegetation from inside of pasture cages and from the pasture ($\delta^{13}C_{cage}-\delta^{13}C_{pasture}$), mean diet–faeces discrimination ($^{13}\Delta_{DF}$) and mean difference of nitrogen content between the C4 and the C3 component ($\%N_{C4}-\%N_{C3}$). The 95% confidence intervals (CI) and error probabilities (P) for July, August, September and the grazing period mean are given. P_1 denotes the significance of a mean larger than 0. P_2 denotes the significance of the $^{13}\Delta_{DF}$ deviating from 0.6‰, the average $^{13}\Delta_{DF}$ compiled from literature.

Month	$\delta^{13}C_{cage}-\delta^{13}C_{pasture}$ (‰)		$^{13}\Delta_{DF}$ (‰)			$\%N_{C4}-\%N_{C3}$ (%)	
	Mean ± CI	P_1	Mean ± CI	P_1	P_2	Mean ± CI	P_1
July	0.31 ± 0.21	0.005	0.39 ± 0.87	0.360	0.646	0.20 ± 0.08	0.002
August	0.46 ± 0.16	≪0.001	0.48 ± 0.55	0.084	0.659	0.61 ± 0.10	≪0.001
September	0.23 ± 0.26	0.08	0.96 ± 0.78	0.017	0.355	0.24 ± 0.10	0.006
Mean	0.34 ± 0.10	≪0.001	0.61 ± 0.41	0.004	0.995	0.35 ± 0.04	≪0.001

by the 270th day of the year (end of September) P_{C3} had dropped to approximately 80%.

On average, the leaf nitrogen content of the C3 component ($\%N_{C3}$) was 0.35% less than that of the C4 component ($\%N_{C4}$) ($P \ll 0.001$; Table 1). This was true until September ($P < 0.01$) when C3 and C4 biomass had the same digestibility. The nitrogen content of herbage on offer (bulk vegetation from the cages) decreased from 1.8 ± 0.1% (CI) in July to 1.7 ± 0.1% (CI) in August and finally to 1.5 ± 0.1% (CI) in September (see Supplementary material).

Seasonal change in $\%N_{faeces}$

The $\%N_{faeces}$ decreased from 1.9% (July) to 1.5% (September) during the grazing period ($P \ll 0.001$; not shown). Following Wang et al. (2009), this corresponds to a digestibility of organic matter of approximately 57% in July and 52% in September. Multiple non-linear regression analysis exhibited no significant influence of P_{C3} ($P > 0.5$) on $\%N_{faeces}$. The day of year was the only significant ($P \ll 0.001$) variable explaining $\%N_{faeces}$.

Apparent selective grazing by sheep

Preferential grazing of one photosynthetic type is reflected in a difference between $\delta^{13}C_{cage}$ and $\delta^{13}C_{pasture}$. The mean $\delta^{13}C_{cage}-\delta^{13}C_{pasture}$ was significantly higher than 0, for July, August and for the grazing period mean (all $P < 0.05$; Table 1; Fig. 2) but not for September ($P > 0.05$; Table 1; Fig. 2). This might indicate a preferential grazing of the C4 component in the early grazing period. The highest mean $\delta^{13}C_{cage}-\delta^{13}C_{pasture}$ of 0.5 ± 0.2‰ (CI) occurred in August, coinciding with C4 peak biomass. Grazing period mean $\delta^{13}C_{cage}-\delta^{13}C_{pasture}$ was 0.3 ± 0.1‰ (CI) (Table 1). Given a 12.1‰ difference in $\delta^{13}C$ between C3 and C4 species (unpublished data of >300 samples), this effect corresponded to a preference of C4 biomass of 4% in August and 2% throughout the grazing period. There was no significant difference, however, in mean
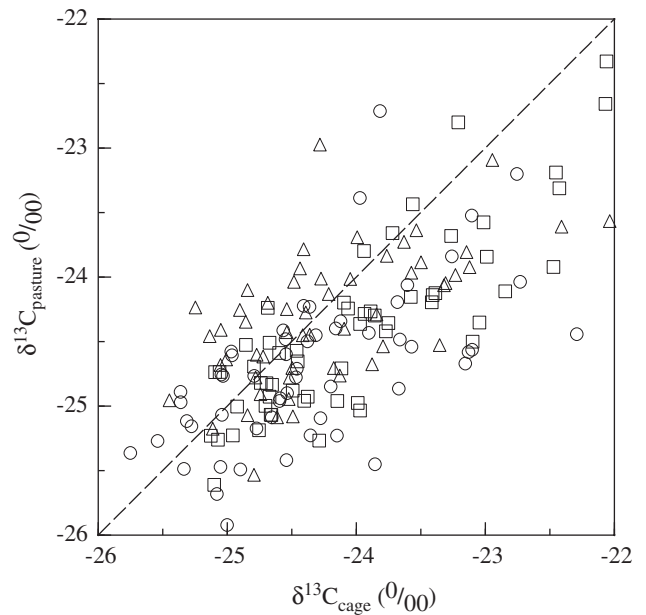

Fig. 2. Relationship between the carbon isotopic composition of vegetation from inside of pasture cages ($\delta^{13}C_{cage}$) and from the pasture ($\delta^{13}C_{pasture}$) for July (○), August (□) and September (Δ). Dashed line denotes the 1:1 line.

$\delta^{13}C_{cage}-\delta^{13}C_{pasture}$ between the stocking rates (all $P > 0.05$, Fig. 3) although $\delta^{13}C_{cage}-\delta^{13}C_{pasture}$ was significantly larger than 0 only for 0.75, 1.5 and 1.875 sheep ha^{-1} year^{-1}.

One might expect a stronger relationship of $\delta^{13}C_{cage}-\delta^{13}C_{pasture}$ with relative feed intake than with stocking rate, as relative feed intake reflects the real opportunity for selection. When relative feed intake varied from 5% to 80%, there was no clear effect on $\delta^{13}C_{cage}-\delta^{13}C_{pasture}$ (Fig. 4). This meant that C4 may have been slightly preferred but this effect did not vary with feed intake.

Within the experiment, relative feed intake covered a wide range. The common stocking rate in the region is slightly above 1 sheep ha^{-1} year^{-1}. For this stocking rate, feed intake ranged between 20% and 60% while $\delta^{13}C_{cage}-\delta^{13}C_{pasture}$ varied from −1.3‰ to +1.0‰. Multiple non-linear regression analysis indicated no
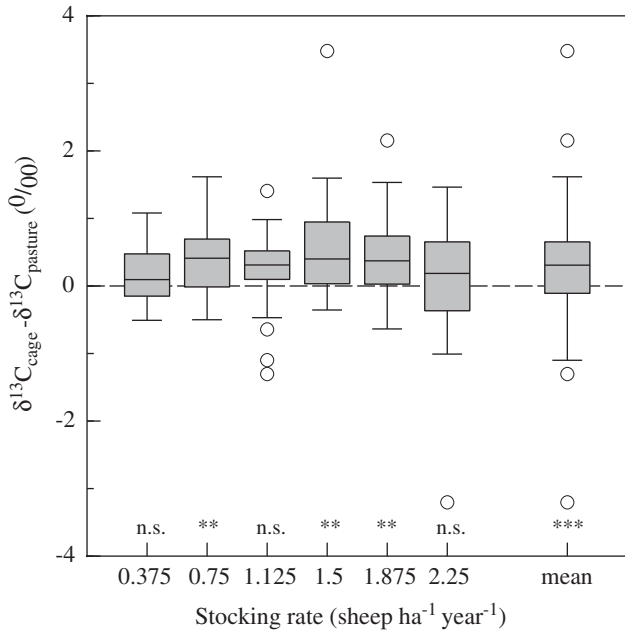
Fig. 3. Box plot for the difference between the isotopic composition of vegetation from the cage ($\delta^{13}C_{cage}$) and from the pasture ($\delta^{13}C_{pasture}$) for all stocking rates and the overall mean. Boxes show the 75% percentile, the median and the 25% percentile; whiskers denote 1.5 times the interquartile range; outliers bigger than that are denoted as circles. The bottom line denotes the significance of the mean deviating from 0.

significant effect of relative feed intake or day of year ($P > 0.5$) on $\delta^{13}C_{cage}$–$\delta^{13}C_{pasture}$.

Seasonal variation in diet–faeces discrimination

The $^{13}\Delta_{DF}$ did not differ among stocking rates (all $P > 0.1$; not shown). The faeces were slightly less depleted in July ($0.4 \pm 0.9‰$ (CI)) compared to diet than in September ($1.0 \pm 0.8‰$ (CI)). Overall the difference between months was 0.6‰. In no month did $^{13}\Delta_{DF}$ differ significantly from the mean 0.6‰ that was observed in a literature compilation of $^{13}\Delta_{DF}$ for pure and mixed C3 and C4 diets (Jones et al. 1979; Coates et al. 1991; Norman et al. 2009). In July and August, the $^{13}\Delta_{DF}$ was not significantly different from 0, but in September and for the grazing period mean the $^{13}\Delta_{DF}$ differed significantly from 0 (Table 1).

Relation between $\delta^{13}C$ of faeces and diet compared to hair

The change in $\delta^{13}C_{faeces}$ or $\delta^{13}C_{diet}$ was not significantly different from the respective change in $\delta^{13}C_{hair}$ (both $P > 0.1$; Fig. 5). This indicated constant faeces–hair ($^{13}\Delta_{FH}$) and diet–hair ($^{13}\Delta_{DH}$) discriminations that were independent of the C3/C4 ratio of ingested biomass. The average $^{13}\Delta_{FH}$ was $-4.3 \pm 0.2‰$ (CI) and
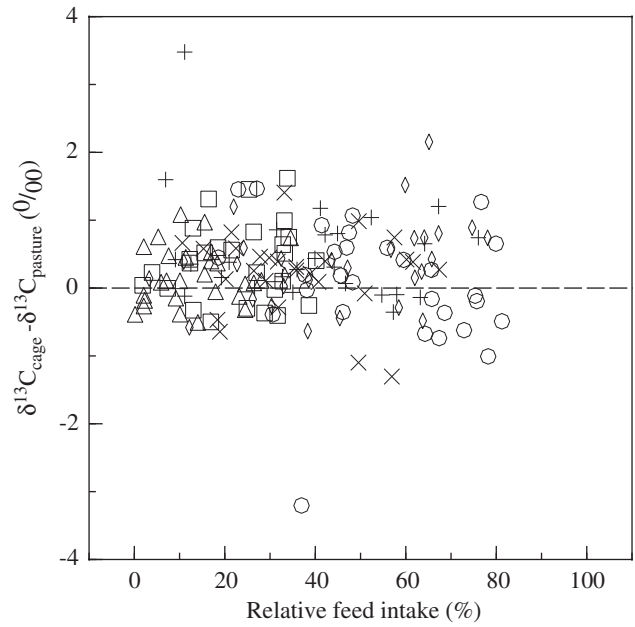
Fig. 4. Relationship between relative feed intake (%) and difference between the isotopic composition of vegetation from the cage ($\delta^{13}C_{cage}$) and from the pasture ($\delta^{13}C_{pasture}$) for all stocking rates: 0.375 ($\triangle$), 0.75 ($\square$), 1.125 ($\times$), 1.5 (+), 1.875 ($\diamond$) and 2.25 ($\circ$) sheep ha^{-1} year^{-1}. The relationships of the individual stocking rates were all not significant ($P > 0.1$).
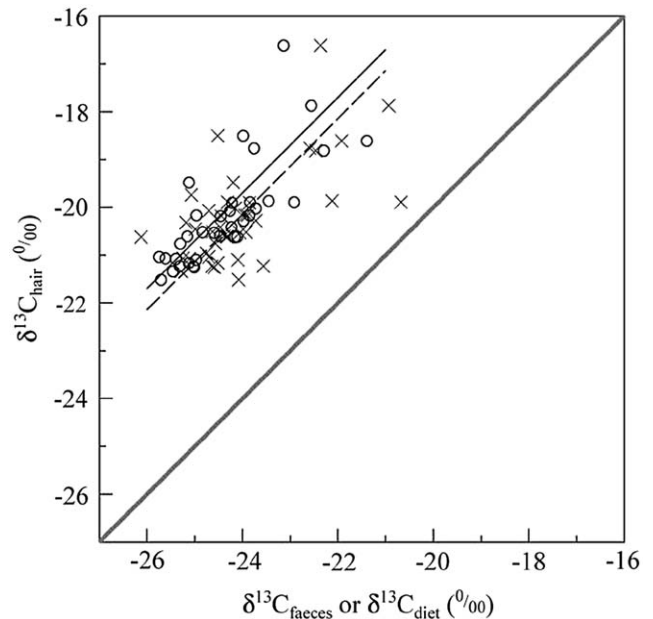
Fig. 5. $\delta^{13}C$ of hair and grazing period mean $\delta^{13}C$ of faeces ($\circ$, solid line) or diet ($\times$, dashed line). The lines denote a constant $^{13}\Delta_{FH}$ of $-4.3‰$ and a constant $^{13}\Delta_{DH}$ of $-3.9‰$, respectively. The bold grey line denotes the 1:1 line.

the average $^{13}\Delta_{DH}$ was $-3.9 \pm 0.3‰$ (CI) (Fig. 5). The latter was not significantly different from an average $^{13}\Delta_{DH}$ of $-3.3‰$ for sheep from literature ($P > 0.1$).

Discussion

Influence of grazing pressure on apparent selection

Our experimental design provided a broad range of stocking rates and yielded little evidence for effects of stocking rate (and related grazing pressure and relative feed intake) on C3 versus C4 selection. This is not an intuitive finding: one might expect that grazers (such as sheep, goat and cattle) select for a certain component when herbage is available in ample supply. Conversely, if competition for feed is high (i.e. when stocking rates increase) then the opportunity for selection is low (e.g. Edwards, Newmann, Parsons, & Krebs 1994; Mobæk, Mysterud, Loe, Holand, & Austrheim 2009). Potential causes for selection are multiple: selection for certain parts of plants driven by taste and odour (Hülber, Ertl, Gottfried, Reiter, & Grabherr 2005), species composition (Jones & Hu 2006; Kausrud, Mysterud, Rekdal, Holand, & Austrheim 2006; Mobæk et al. 2009), site productivity (Kausrud et al. 2006; Mobæk et al. 2009) or dietary quality (Garcia, Carrere, Soussana, & Baumont 2003). However, we found no effect of stocking rate and relative feed intake on the relationship between the $\delta^{13}C$ of ingested feed, meaning that variable opportunities for selection had actually no effect on selection for C3 and C4. Hence, the contrast in attributes that drive selection between C3 or C4 species was apparently too small to cause an effect in this typical Inner Mongolian steppe. This finding agrees with those of Hodgkinson, Terpstra, and Müller (1995) who examined the defoliation pattern of two contrasting C3 and C4 grasses in semi-arid and arid Australian woodland and found no clear effect of grazing pressure on selection. Further, Schiborra et al. (2009) compared the organic matter digestibility of herbage on offer and herbage ingested in the same experiment and found no evidence of an effect of grazing intensity on selection.

The small difference between $\delta^{13}C_{cage}$ and $\delta^{13}C_{pasture}$ ($+0.3 \pm 0.1$‰ CI) was barely significant owing to the large number of data points ($n = 177$) and such a small effect could be due to a number of reasons. Most likely the effect is an artefact of the sampling, e.g. when clipping height did not exactly match the biting height (Davies, Milton, & Lloyd 1950; Griffiths, Hodgson, & Arnold 2003). Importantly, the lack of any correlation to parameters that indicate the opportunity of selection like stocking rate or relative feed intake indicates that the difference does not originate from selection.

Influence of growth initiation and digestibility

Several studies have reported an inferior digestibility of C4 species compared to C3 species (e.g. Barbehenn et al. 2004; Norman et al. 2009). But digestibility also decreases strongly with plant age (Wilson & Hacker 1987; Buxton 1996). As the C4 species of the Inner Mongolian grassland initiate growth about 30–50 days later than C3 plants (Bai et al. 2004), the C4 species likely have a relatively high digestibility (see Milchunas, Varnamkhasti, Lauenroth, & Goetz 1995). This agrees with the present results in that the average nitrogen content of the C4 component was actually higher than that of the C3 component and that %N of herbage on offer decreased significantly from August to September ($P \ll 0.001$). In the same experiment, Schiborra et al. (2009) analyzed in vitro digestibility of organic matter and found the same effects.

However, a significant influence of differential digestibility on $^{13}\Delta_{DF}$ was not evident. The $^{13}\Delta_{DF}$ was not significantly different from the mean $^{13}\Delta_{DF}$ of 0.6‰ which was observed when sheep were fed pure and mixed C3 or C4 diets (Coates et al. 1991; Jones et al. 1979; Norman et al. 2009). Also, $^{13}\Delta_{DF}$ was near-constant during the grazing period.

Differences in digestibility between C3 and C4 components should also influence $^{13}\Delta_{DH}$. As digestibility influences $^{13}\Delta_{DF}$ and $^{13}\Delta_{DH}$ in opposite directions (Fig. 1), the effect of differential digestibility on $^{13}\Delta_{FH}$ should be even more pronounced ($^{13}\Delta_{FH}$ is equal to the difference between $^{13}\Delta_{DF}$ and $^{13}\Delta_{DH}$). $^{13}\Delta_{FH}$ was -4.3‰, close to the expected value (-3.9‰) that was derived from published estimates of $^{13}\Delta_{DF}$ (mean -0.6‰) and $^{13}\Delta_{DH}$ (mean -3.3‰). Furthermore, there was no systematic variation in the $^{13}\Delta_{FH}$ related to an increase in the proportion of C4. Thus there was no evidence for effects of differential digestibility on $^{13}\Delta_{DF}$ or $^{13}\Delta_{DH}$.

$\delta^{13}C$ of faeces and hair as a tool to estimate the C3/C4 ratio of vegetation

The above findings are important for the estimation/reconstruction of C3/C4 ratios of grassland from the $\delta^{13}C$ of sheep faeces and wool. To a close approximation, sheep grazed C3 and C4 vegetation non-selectively, and both components had similar digestibility, yielding approximately constant $^{13}\Delta_{DF}$ and $^{13}\Delta_{DH}$. Thus, the $\delta^{13}C$ of grassland vegetation could be predicted from that of faeces or wool by applying a constant discrimination, which was related to constant animal properties. Notably, the integration over time (and, hence, space if animals range unconfined) usually differs between faeces (a few days) and hair (a few months to one year). This can be used to estimate C3/C4 ratios at different scales from $\delta^{13}C$ by analyzing the appropriate tissue. Faeces integrate the diet of less than one week (Raymond 1948), which corresponds to the area grazed in less than 1 week, while hair integrates the isotopic signal of the diet ingested between two shearings. Thus, hair provides a much higher degree of spatio-temporal integration

(or averaging) and is more suitable for the assessment of yearly mean C3/C4 composition, when the latter fluctuates seasonally or spatially within the grazing area of a flock of sheep. Evidently, a time series of hair samples would be useful for the reconstruction of long-term changes in the C3/C4.

Conclusions

The C3/C4 ratio of the Inner Mongolian grassland as indicated by community $\delta^{13}C$ varied in space and time due to the differential growth initiation of C3 and C4 plant species. Instantaneous sampling of vegetation did not reflect the C3/C4 ratio of annual growth under these conditions. The $\delta^{13}C$ of faeces and hair integrated over the grazing area and period, with integration times differing for hair and faeces. Neither faeces nor hair were influenced by selective grazing of sheep within a reasonable degree of uncertainty. The mean $^{13}\Delta_{DF}$ of $0.6\pm0.4‰$ (CI) and the mean $^{13}\Delta_{DH}$ of $-3.9\pm0.3‰$ (CI) in this study were close to published estimates obtained with C3 or C4 feeding. $^{13}\Delta_{FH}$ of $-4.3\pm0.2‰$ (CI) was near-constant, although this parameter should respond strongly to selective grazing and differences in digestibility between the C3- and C4-component. This indicated that discrimination was not influenced by differential digestibility of C3 and C4 species within uncertainty. Our findings demonstrate that $\delta^{13}C_{faeces}$ and $\delta^{13}C_{hair}$ are suitable proxies for the C3/C4 ratio of Inner Mongolia grassland.

Acknowledgments

Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.baae.2009.10.007.

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