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Stable isotopes for determining carbon turnover in sheep feces and blood ☆

M.B. Martins [a,*,1], C. Ducatti [b], C.L. Martins [a], J.C. Denadai [b], A.S. Natel [a], C.R. Souza-Kruliski [b], M.M.P. Sartori [b]

[a] Department of Animal Production, State University Julio de Mesquita Filho—UNESP, Botucatu, SP 18.600-000, Brazil
[b] Department of Physics and Biophysics, Stable Environmental Isotope Center, State University Julio de Mesquita Filho—UNESP, Botucatu, SP 18.618-000, Brazil

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ABSTRACT

The objective of this work was to evaluate carbon turnover and half-life in feces and blood from sheep fed on C_3 and C_4 plant diets, using the stable isotope technique. Eight Santa Ines sheep were randomly distributed between two treatments: the first consisted of animals that were fed alfalfa hay, and the second consisted of animals that were fed corn silage only. Function of time was used to measure carbon turnover rate. At steady state, the half life for carbon isotopic enrichment between food and feces is 1.2 and 1.0 days for alfalfa hay and corn silage treatments, respectively. For blood data collection the time was insufficient to reach the isotope equilibrium level, indicating a slow carbon exchange between diet and blood. It is concluded that blood has a slow turnover, indicating the isotope signal for the former diets. Feces, by contrast, have a quick turnover, indicative of their recent diets.

1. Introduction

The stable isotope technique has practical potential for tracing food products of animal origin. There is a growing demand for qualitative information on food given to animals, as reflected by the increasing interest of consumers relative to the "green" image for products of animal origin (Prache et al., 2005). The isotope technique has recently been proposed as a tool for animal product authentication (Bahar et al., 2008; Chesson et al., 2008; Heaton et al., 2008), for certification of the geographic origin and types of ovine feed (Piasentier et al., 2003), and as an evaluation of conventional and organic production systems for beef (Schimidt et al., 2005), among others.

The suitability of this technique for complementing and providing greater support for meat traceability programs is of paramount importance, since the exporting market increasingly demands the absence of products of an animal origin, of any nature, in feed, specially destined for the production of meat. Through the use of stable isotopes, which occur naturally as dietary indicators, information can be obtained on diets made over both short and long term periods, depending on the type of tissue evaluated. However, to obtain such information there is a need to carry out studies related to the isotopic assimilation of organic matter in different tissues of animals (turnover).

According to Ducatti (2007), isotopic turnover is the continuous renewal of chemical elements and consequently their isotopes which make up the bodily tissue or the organism as a whole. The turnover can occur through tissue renewal resulting from the process of synthesis and degradation in adult tissue and/or the tissue growth itself under formation (isotopic assimilation). All body substances, whether organic or inorganic metabolites are subject to turnover (Hetenyi et al., 1983).

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* Corresponding author. Tel.: +55 1514 3811 6359; fax: +55 1514 38151117.
E-mail addresses: mar_buosi@hotmail.com (M.B. Martins), ducatti@ibb.unesp.br (C. Ducatti).
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The choice of what type of animal tissue or organ should be collected for carbon isotopes analysis may influence conclusions concerning the diet. This is due to significant variations in the values of $\delta^{13}C$ between biochemical fractions and between the different tissues in the animal's organism (Deniro and Epstein 1978). Analysis of feces and ruminal content provide indications of the diet ingested over a short period, ranging from a few hours, in the case of insects, to several days, for large herbivore mammals (Tieszen et al., 1983).

The isotopic composition for a given tissue depends on the tissue's carbon turnover during that period. In general, the most metabolically active tissues have a faster turnover than less metabolically active tissues. Therefore, the amount of time between changing the diet and the manifestation of same in the animal tissue varies according to the tissue which is being analyzed. The evaluation of tissue of distinct metabolisms can be indicative of changes in the animals' diet feed during their lifetime, detecting in unscrupulous cases, if the pre-slaughter diet has been manipulated in an attempt to hide the use of prohibited products, for example, ground/powdered feed of an animal origin in the diet of the animals, in order to obtain certification.

Bahar et al. (2005) studying (lipid free) muscle tissue turnover in cattle fed on corn silage (C_4) in place of grass silage (C_3), observed that after 167 days of the experiment, tissue turnover was not complete, reflecting $\delta^{13}C$ of the old diet. This demonstrates that muscle tissue in cattle is not appropriate for short and recent changes in diet.

The turnover rate evaluates the rate at which $\delta^{13}C$ is replaced and/or incorporated into different animal tissues. In the process of authenticating an animal-based product, the goal is to find a tissue or fraction that can reflect former diets, thus making it possible to detect the inclusion of any banned ingredients, even if introduced only during the early life stages of the animal. To that end, a slow-metabolism tissue must be found that preserves the isotopic signal of the initial diet for a longer period of time, and a tissue or fraction that can quickly replace the carbons of the old diet with those of the new one, thus indicating whether or not there was a change in diet during rearing, and when it occurred. Therefore, it becomes necessary to study two tissues or fractions with different turnover rates, so that the obtained results can serve as benchmarks for future authentication studies.

There is currently scarce information on turnover rates in different ovine tissues. Therefore, the objective of this paper was to evaluate carbon turnover and half-life in the feces and blood of sheep fed on plant diets C_3 and C_4, using the stable carbon isotope technique.

2. Material and methods

The field experiment was conducted at UNESP—Botucatu Campus, in accordance with the ethics committee norms (Protocol no. 78/2008-CEEA) for the College of Veterinary Medicine and Animal Husbandry, in the experimental digestibility installations of the Department of Animal Improvement and Nutrition at the Lageado Farm over a period of 150 days.

For this study, eight four month old Santa Ines sheep were used, with an average live weight of 28.6 ± 1.13 kg, from C_4 type grassy pastures. These sheep were randomly distributed between two separate feed treatments areas, 2×2 m^2 each, using drink and feed type troughs.

The animals went through a 45-d period of adaptation (pre-experimental period), receiving a diet consisting of 50% corn silage and 50% alfalfa hay, provided twice a day, at 9 am and 4 pm. The quantity of food provided was calculated at 4% of the sheep live weight in dry matter. The pre-experiment diet was used in order to help animals adapt to the new diets and mark the tissues in question with intermediate isotopic signals, as the animals had formerly grazed exclusively on C_4 pastures.

After the adaptation period, the animals were randomly distributed into two treatments (with 4 experimental sheep for each treatment). C_4-SM treatment consisted of animals that received only corn silage (C_4) and C_3-FA treatment for animals that received only alfalfa hay (C_3), both of which were provided freely. The nutritional values of the diets given to the sheep are shown in Table 1, as are the measured $\delta^{13}C$ values.

Samples were taken on the following days: 0, 1, 2, 4, 7, 10, 12, 14, 18, 21, 28, 35, 42, 49, 56, 63, 70, 77, 84, 91, 95, 100, 103 and 104. Sampling was concentrated on the initial days due to faster carbon exchange rates.

Blood samples were taken from the animals' jugular veins, packed into eppendorfs and frozen. Feces were collected directly through a rectal ampule, packed into plastic bags and then duly identified and frozen.

Samples of both blood and feces were collected, as according to Jones et al. (1979), there is contamination of the feces with endogenous material, which could be one of the possible causes for the differences in the $\delta^{13}C$ values taken from the diet and fecal material. In the case of blood this contamination does not occur. Furthermore, in the case of feces, this is understood to be non-assimilated feed and in the case of blood it is expected to encounter assimilated nutrients.

The isotopic analyses were conducted at the Stable Environmental Isotope Center at UNESP—Botucatu Campus.

In order to carry out the isotopic analysis on the experimental diets and feces, the samples were thawed out, dried in a forced draft dryer at 56 °C over a period of

Table 1
Nutritional values based on dry matter and isotopic values of experimental diets.

%	50% alfalfa hay + 50% corn silage	Alfalfa hay	Corn silage
Dry matter	49.03	82.94	34.32
Crude protein	12.68	16.65	8.39
Ether extract	4.81	3.46	6.49
Ash	5.78	9.36	3.71
Neutral detergent fiber	44.25	57.45	42.85
Acid detergent fiber	32.03	47.53	26.07
Cellulose	26.26	36.53	23.42
Lignin	5.77	11.00	2.65
Total digestible nutrients	63.85	58.65	71.81
$\delta^{13}C$ (‰)	−22.40	−31.15	−13.67

48 h and cryogenically ground (cryogenic grinder model Spex—6750 freezer/mill, Metuchen, EUA), to −196 °C, according to the methodology described by Carrijo et al. (2006), Denadai et al. (2008) and Móri et al. (2007). During grinding, approximately 2.0 g of the sample was collected in a poly-carbon flask together with a magnetic bar, which was duly closed and immersed in liquid nitrogen. The impact between the sample and the magnetic bar under an oscillating magnetic field of 15 impacts/s, pulverized the sample. The program used in the grinding of the samples consisted of first stage pre-freezing for 1 min and the second stage of freezing and grinding for 5 min. This procedure permitted obtaining particles with a granulometer of less than 60 μm. After grinding, about 50–60 μg of the samples was weighed and packed into tin capsules. The blood samples were defrosted and pipetted (0.3 μl) into tin capsules.

The tin capsules were placed in the EA 1108 CHN Elementary Analyzer attached to a DELTA-S (Finnigan Mat) mass spectrometer at the UNESP Bioscience Institute's Stable Isotope Center, Botucatu Campus. Results were expressed in $\delta^{13}C$ notation in relation to the Peedee Belemnite (PDB) standard, with an analysis error of around 0.2‰ and calculated by Eq. (1):

$$\delta^{13}C_{(sample,\ standard)} = [(R_{sample}/R_{standard}) - 1] \times 10^3 \quad (1)$$

Where: $\delta^{13}C$ is the enrichment related to ratio $^{13}C/^{12}C$ of the sample, in relation to the PDB standard; R is the isotopic ratio ($^{13}C/^{12}C$) of the sample and the standard.

In order to measure carbon turnover (isotope substitution) in feces and blood over a specific time period, the exponential function of time was used expressed by Eq. (2) (Ducatti et al., 2002), using Origin® 6.0 Professional (Microcal Software Origin® 6.0 Professional, 1999):

$$\delta^{13}C(t) = \delta^{13}C(f) + [\delta^{13}C(i) - \delta^{13}C(f)]e^{-kt} \quad (2)$$

where $\delta^{13}C(t)$ is the isotope enrichment of feces or blood at any time (t); $\delta^{13}C(f)$ is the isotope enrichment of feces or blood at equilibrium, or final condition; $\delta^{13}C(i)$ is the isotope enrichment of feces or blood, at initial condition; t is the time (in days) since diet replacement.

Isotope fractionation was expressed by the difference between the δ‰ of the tissue and of the diet, as per Eq. (3)

$$\Delta = \delta‰_{tissue} - \delta‰_{diet} \quad (3)$$

The positive value of the large delta (Δ) indicates that the tissue is richer in Carbon-13 than the diet (Hobson and Clark, 1992).

In order to determine the exchanged time for carbon atoms, Eq. (4) was used:

$$t = (-1/k)\ln(1-F) \quad (4)$$

where F is the fraction of exchanged atoms.

In this case, F was substituted for 0.5 (half-life) and 0.99 (99% of exchanged atoms).

3. Results and discussion

The $\delta^{13}C$ values in feces and blood at the beginning and at the end of the experimental period are shown in Table 2. It can be verified that the isotopic composition of the feces directly reflects the animals' diets after a pre-experimental period, with diet-tissue isotopic fractionation of −0.32‰. The blood values did not reach the isotopic signal stabilization (Fig. 1). This is believed to have occurred due to a slower carbon turnover because the 45-d adaptation period was insufficient for a complete carbon turnover in the blood, which still reflected the isotopic signal of the animals' former diet (animals came from a C_4 plant photosynthetic cycle feeding system).

Table 2
Average values of $\delta^{13}C$ for the diets, feces and blood in accordance with the food under study.

Diets	$\delta^{13}C$ (‰)		
	Diets	Feces	Blood
Pre-experimental diets	−22.40 ± 0.10	−22.72 ± 0.06[a]	−17.40 ± 0.36[a]
Corn silage	−13.67 ± 0.10	−14.54 ± 0.14	−13.67 ± 0.70
Alfalfa hay	−31.15 ± 0.10	−31.60 ± 0.37	−24.56 ± 0.35

[a] Values of $\delta^{13}C$ of the feces and blood after the pre-experimental period.

After the experimental period, the ^{13}C in the feces was reduced relative to the diet in both treatments, although in treatment C_3-FA, this effect was smaller (diet-tissue isotope fractionation of −0.45‰). This data agrees with that of Norman et al. (2009) who found lower $\delta^{13}C$ values for the feces compared to that of the diet. In the case of blood, the $\delta^{13}C$ values of the analyzed samples corresponded only to the C_4 plant $\delta^{13}C$ values. The $\delta^{13}C$ values of the blood in animals fed with C_3 plant did not reach the isotopic signal stabilization. However, the values were found to be indicative of the type of diet that the sheep were being fed. This fact was possibly due to the existing difference in $\delta^{13}C$ values between plants C_3 and C_4. In C_3 plants, the $\delta^{13}C$ value varies between −22 and −34‰, depending on the species and plant parts analyzed, and in C_4 plants, this value ranges between −9 and −16‰ (Vogel, 1993). Considering an isotopic difference of about 16‰ between the $\delta^{13}C$ value of plants with a C_3 and C_4 photosynthetic cycle, it is possible to characterize the animal's diet by means of carbon isotopic analysis of blood.

In accordance with the results obtained in the isotopic analyses, the change in $\delta^{13}C$ values in the blood of the sheep over a period of time was observed to occur gradually, whereas the change in $\delta^{13}C$ values in feces occurred quickly. According to De Smet et al. (2004), Dalerum and Angerbjörn (2005) and Gratton and Forbes (2006), animal tissues or endogenous matter reflect carbon integration over different periods of time, i.e., the most metabolically active tissues have a faster turnover than less metabolically active tissues.

The evolution of time on carbon isotope composition in feces and blood for experimental treatments C_3-FA and C_4-SM is illustrated in Fig. 1.

The $\delta^{13}C$ value of feces for both treatments (Fig. 1a and b) responded exponentially, which is due to the diet's quick carbon turnover, in accordance with the findings of Gratton and Forbes (2006) and Sponheimer et al. (2006).

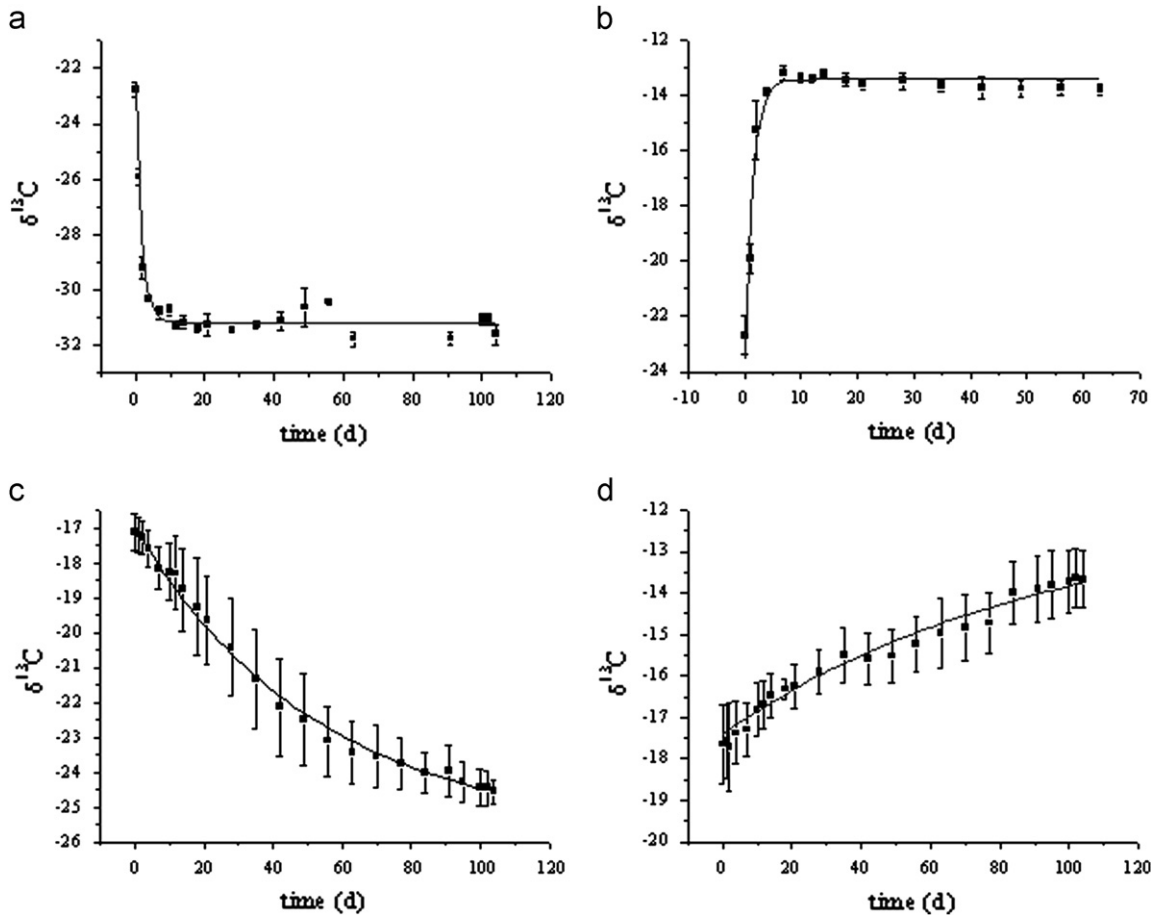

Fig. 1. Carbon isotope dilution curves (average of animals) for sheep feces in Treatment C_3-FA (a); for feces in Treatment C_4-SM (b); for sheep blood in Treatment C_3-FA (c) and blood in Treatment C_4-SM (d), as a result of the evaluation interval.

The feces demonstrated a quick transition of the food through the gastrointestinal tract and can be considered to be indicative of that which has been recently consumed by the animal. In these cases they reached the new isotopic steady state quickly. At the beginning of the experimental period, sheep feces (average for animals by experimental treatment) presented carbon isotopic ratios of −22.76‰ and −22.68‰, for C_3-FA and C_4-SM treatments, respectively; these values were similar to those of in the mixed diet given during the animals' adaptation period, which comprised of 50% alfalfa hay+50% with silage (Table 2). At the end of the experiment, the carbon isotopic ratio reaches values of −31.60‰ and −14.54‰. Therefore, sheep fed with the C_3 diet could be differentiated from those fed with C_4. The final carbon isotopic values for animal feces for both treatments were close to the isotopic values of the experimental diets, with an apparent isotopic fractionation of −0.45‰ and −0.87‰ for C_3-FA and C_4-SM treatments, respectively. According to Jones et al. (1979) and Tieszen et al. (1983) the exogenous tissues or matter can be relatively impoverished or enriched by $\delta^{13}C$ in relation to the diet, due to isotopic fractionation. These isotopic fractionation values agree with those found by Jones et al. (1979) for cattle (interval between −0.4 and −2.0‰).

Table 3
Exponential equations for isotopic dilutions as a function of time for animal feces in C_3-FA treatment with their respective coefficients of determination (R^2).

Animal	Equations	R^2
An. 1	$\delta^{13}C = -31.15 + 8.66e^{-0.65t}$	0.96
An. 2	$\delta^{13}C = -31.25 + 8.27e^{-0.55t}$	0.95
An. 3	$\delta^{13}C = -31.28 + 8.62e^{-0.55t}$	0.96
An. 4	$\delta^{13}C = -31.08 + 8.69e^{-0.62t}$	0.97
Mean	$\delta^{13}C = -31.17 + 8.52e^{-0.56t}$	0.92

Exponential equations as a function of time and coefficients for determining feces from C_3-FA and C_4-SM treatments are shown in Tables 3 and 4 and are in accordance with those models proposed by Ducatti et al. (2002) and Zuanon et al. (2006). These authors confirmed that this technique can be used for animals during their growth phase, as was the case in this study, as according to Millward (1989), generally speaking, the mechanisms

Table 4
Exponential equations for isotopic dilutions as a function of time for animal feces in C_4-SM treatment with their respective coefficients of determination (R^2).

Animal	Equations	R^2
An. 1	$\delta^{13}C = -13.45 - 9.00e^{-0.47t}$	0.95
An. 2	$\delta^{13}C = -13.43 - 10.29e^{-0.66t}$	0.98
An. 3	$\delta^{13}C = -13.45 - 9.66e^{-0.63t}$	0.97
An. 4	$\delta^{13}C = -13.56 - 9.57e^{-0.80t}$	0.94

Table 5
Half-life values calculated (days) for animal feces for experimental treatments starting the day of diet substitution.

Animal	C_3-FA[a]	C_4-SM[b]
An. 1	1.1	1.5
An. 2	1.3	1.0
An. 3	1.3	1.1
An. 4	1.1	0.9
Mean	1.2	1.0

[a] 50% C_3+50% C_4 for 100% C_3.
[b] 50% C_3+50% C_4 for 100% C_4.

for regulating the muscle tissue in fish are comparable to mammal animals.

Carbon half-life in feces was calculated using exponential equations (Table 5), which was 1.2 and 1.0 days for C_3-FA and C_4-SM treatments, respectively. These values indicate the speed that feces take to reflect the new diet, a fact that can be associated with the digestion rate through the gastrointestinal tract.

The substitution times for 99% of the feces carbon atoms relative to those on the new diets (experimental treatments), i.e., the time needed for the stabilization of the isotopic signal in which the carbon values in the feces reached a stable isotopic level were 8.3 and 6.5 days for C_3-FA and C_4-SM treatments, respectively. Jones et al. (1979) investigated changes in $\delta^{13}C$ values for calf and cow feces as a result of different diets. After the change from the C_4 to the C_3 diet, a complete turnover was observed in 6 days. In an experiment conducted by Norman et al. (2009), the substitution time for 95% of $\delta^{13}C$ in ovine feces occurred 6 days after changing from a C_3 diet to a C_4 diet. These results were similar to this study as the substitution time for $\delta^{13}C$ of the feces by diet C_4 was between 6.5 and 8.3 days.

Observing the blood isotopic dilution curves for the C_3-FA treatment (Fig. 1c), we see that the $\delta^{13}C$ values for blood did not reach the new isotopic equilibrium level, indicating that there was a slower carbon turnover, when compared to the carbon turnover in the feces. Although, the final isotopic value of the blood in the animals under treatment C_4-SM (Table 2) was found to be similar to that of the diet, the isotopic dilution curve (Fig. 1d) showed that on average, the isotopic equilibrium level was not reached.

The blood samples could be used to distinguish the sheep fed with diet C_3 from the sheep fed with diet C_4. At the end of the experimental period, the isotopic values found in the blood of the sheep receiving corn silage was $-13.67‰ \pm 0.70$, whereas the blood of sheep fed alfalfa hay was $-24.56‰ \pm 0.35$. The isotope difference of 10.89‰ between $\delta^{13}C$ blood values for treatments C_3 and C_4 made it possible to differentiate the sheep fed with C_3 plant from those fed with C_4. This result agrees with De Smet et al. (2004) data, on young bulls fed with a C_3 plant diet, or receiving a mixture of C_3 and C_4 plants (about 59% of C_4). These authors observed a clear carbon isotopic difference between treatments after 70 days in total blood and plasma samples but, they did not observe a complete turnover after this period. Bahar et al. (2005) studying lipid free muscle tissue turnover (Longissimus thoracis et lumborum) in cattle fed with corn silage (C_4) in place of grass silage (C_3), verified that after 167 days of the experiment, tissue turnover was not complete. All together, these results demonstrate that the choice of animal tissue or organ may have consequences on the dietary reconstitution because tissues with a quick metabolism indicate recent diets, whereas those with a slow substitution rate indicate feed ingested earlier.

The half-life values for blood could not be calculated, because from the exponential equation for isotopic dilution proposed by Ducatti et al. (2002), the half-life could only be calculated if the $\delta^{13}C$ values for the analyzed tissue reached the isotopic equilibrium level.

From the results of the blood turnover, it is implied that the carbon turnover rate varies with the animal's size under study. In addition to this animals which are ruminants possess a different physiological digestive system to monogastric animals, which can also account for the difference in the carbon turnover rates.

4. Conclusions

From the differences analyzed between the feces and the blood, it was demonstrated that each tissue type has a specific turnover rate.

Female Santa Ines sheep fed on a C_3 plant diet can be differentiated from females fed on a C_4 diet, through samples taken from their feces and blood.

The measure of turnover in feces represents the passage of food through the gastrointestinal tract, while the measure of turnover in blood indicates the assimilation of the isotopic signal in the diet.

Conflict of interest statement

We declare that there aren't conflict of interests in this project.

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