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Box-modeling of $^{15}N/^{14}N$ in mammals

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Abstract The $^{15}N/^{14}N$ signature of animal proteins is now commonly used to understand their physiology and quantify the flows of nutrient in trophic webs. These studies assume that animals are predictably ^{15}N-enriched relative to their food, but the isotopic mechanism which accounts for this enrichment remains unknown. We developed a box model of the nitrogen isotope cycle in mammals in order to predict the $^{15}N/^{14}N$ ratios of body reservoirs as a function of time, N intake and body mass. Results of modeling show that a combination of kinetic isotope fractionation during the N transfer between amines and equilibrium fractionation related to the reversible conversion of N-amine into ammonia is required to account for the well-established ≈4‰ ^{15}N-enrichment of body proteins relative to the diet. This isotopic enrichment observed in proteins is due to the partial recycling of ^{15}N-enriched urea and the urinary excretion of a fraction of the strongly ^{15}N-depleted ammonia reservoir. For a given body mass and diet $\delta^{15}N$, the isotopic compositions are mainly controlled by the N intake. Increase of the urea turnover combined with a decrease of the N intake lead to calculate a $\delta^{15}N$ increase of the proteins, in agreement with the observed increase of collagen $\delta^{15}N$ of herbivorous animals with aridity. We further show that the low $\delta^{15}N$ collagen values of cave bears cannot be attributed to the dormancy periods as it is commonly thought, but inversely to the hyperphagia behavior. This model highlights the need for experimental investigations performed with large mammals in order to improve our understanding of natural variations of $\delta^{15}N$ collagen.

Keywords Ammonia · Cave bear · Nitrogen · Stable isotope · Urea recycling

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Introduction

Since the pioneering works done by DeNiro and collaborators (DeNiro and Epstein 1981; Schoeninger and DeNiro 1983, 1984), the $^{15}N/^{14}N$ isotopic ratio of bone collagen was extensively used to reconstruct extant and past trophic web structures (e.g., Minagawa and Wada 1984; Balter et al. 2002; Post 2002), paleodiet (e.g., Richard et al. 2003), paleoenvironments (e.g., Iacumin et al. 2000; Jones et al. 2001; Stevens and Hedges 2004), and migration pathways (Koch et al. 1995; Hobson 1999).

A ^{15}N-enrichment between an organism and its diet ($\Delta^{15}N$) was systematically documented (e.g., DeNiro and Epstein 1981; Ambrose 2000) with a $\Delta^{15}N$ between the lean mass and the diet ($\Delta^{15}N_{lm-d}$) usually ranging from 3 to 5‰ vs AIR (e.g., Minagawa and Wada 1984; Schoeninger and DeNiro 1984; Ambrose 2000). However, the processes which account for this ^{15}N-enrichment remain still misunderstood. Experimental studies performed with microorganisms have shown that the organic residues during peptide bond hydrolysis, transamination, and amino acids synthesis are ^{15}N-depleted in comparison to the product of the reaction due to the

higher reactivity of ^{14}N (Macko et al. 1986, 1987; Silfer et al. 1992). Therefore, Gröcke et al. (1997) and Ambrose (2000) proposed that the positive $\Delta^{15}N$ could result from kinetic isotope fractionation during the preferential excretion of ^{14}N-urea, corresponding to a relative ^{15}N retention in the body. The fact that urine is ^{15}N-depleted relative to diet seems to support this hypothesis (e.g., Sutoh et al. 1993; Sponheimer et al. 2003). However, fecal N is usually ^{15}N-enriched relative to diet and could represent 40% of total excreted N (references in Table 1). Thus, the $\delta^{15}N$ of the total excreta should not be systematically ^{15}N-depleted relative to diet (Sponheimer et al. 2003).

Both production and excretion of aqueous ammonia (NH_3), which are important parts of the N metabolism, were never taken into account in previous studies. Ammonia is the end-member product of the amino acids catabolism and could also be released from urea by the microorganisms of the digestive tract (e.g., Wright 1995). However, ammonia is a highly toxic compound and glutamine synthesis is the most important pathway for ammonia detoxification. Glutamine, which is formed by equimolar amounts of glutamate and ammonia, is a non-essential amino acid and constitutes approximately 25% of the whole body free amino acid pool (Table 1). Ammonia is bounded to glutamine under a protonated NH_3^+ group. The change in the N oxidation state from an N-amine group of an amino acid ($R\text{-}NH_2$) to the $R\text{-}NH_3^+$ form is accompanied by ^{15}N equilibrium fractionation (Rishavy and Cleland 1999). This ^{15}N equilibrium fractionation (noted hereafter $^{15}\alpha_{r \to p}$) is equal to the $(^{15}N/^{14}N)_r/(^{15}N/^{14}N)_p$ ratio where the subscripts r and p stand for the reactant and the product, respectively. Hermes et al. (1985) reported equilibrium isotope fractionation factors of 1.0163 between $R\text{-}NH_2$ and aqueous NH_3 and 1.0333 between $R\text{-}NH_3^+$ and aqueous NH_3. These considerations likely add new constraints on the understanding of N isotope compositions of mammals.

A time-dependent box-model is developed to calculate the $^{15}N/^{14}N$ evolution of the main reservoirs involved in the N metabolism of mammals. Nitrogen fluxes and reservoirs are scaled to body mass in order to use the same model for a variety of mammals by means of allometric relationships. We propose that the measured ^{15}N-enrichment between the lean mass and the diet is the result of two competing isotopic fractionations associated with transamination and N transfer during the NH_3 cycle. Finally, some revised interpretations of debated biogeochemical case studies are proposed.

Description of the N cycle

The N cycle is formulated to closely represent the main transfers and biochemical reactions of the mammalian metabolism (Fig. 1). All the fluxes are scaled as a function of body mass, allowing the transposition of the model to any mammal (Table 1). The major N intake comes from dietary proteins [flux 1, noted thereafter (**f1**)] which is released toward the gastro-intestinal tract (box 1, noted thereafter *b1*). Moreover, the total N intake must take into account pancreatic secretion and intestinal desquamation (**f10**) having an endogenous origin (Tomé and Bos 2000). During these transfers, the proteins are hydrolyzed into free amino acids which are incorporated into the circulating free amino acid subreservoir (**f5** and *b2*), the remaining proteins being excreted in the form of feces (**f11** and *b7*). The proteins are synthesized from free amino acids (**f8**) constituting the lean mass reservoir (*b4*). Protein degradation leads to the formation of free amino acids that are incorporated into the circulating pool (**f9**) and the formation of residual aqueous ammonia (NH_4^+). Aqueous ammonia that is derived from peripheral tissues is carried as a non-ionizable species (**f7**) with glutamate to form glutamine because of its high toxicity (*b3*; Young and Ajami 2001). The N cycling is achieved through urea synthesis (**f3** + **f6**), hydrolysis (**f4**) and excretion (**f13**). The synthesis of urea occurs in the liver (*b5*) through a series of reactions known as the Krebs–Henseleit Cycle. The net balance for the formation of 1 mol of urea is the consumption of 1 mol of ammonia and 1 mol of amino acid (Mathews et al. 1999). The stoechiometry of this reaction thus imposes to equalize fluxes **f3** and **f6**. Synthesized urea is then partly brought to the kidney where it is excreted by urine (**f13**) along with a fraction of NH_4^+ (**f12**). The remaining urea is transferred to the gastro-intestinal tract lumen (**f4**) where it is hydrolyzed by the urease of microorganisms. The hydrolysis of urea by microorganisms leads to the formation of ammonia which is reabsorbed through the intestine (**f2**) and reincorporated essentially into glutamine (*b3*). If the biomass of intestinal microorganisms is assumed to be at steady-state, the ammonia assimilation for metabolism of microorganisms should balance the ammonia formation due to protein degradation (Wright 1995). Consequently, the return of ammonia toward *b3* (**f2**) equals **f4**. All these processes (**f2**, **f3** and **f4**) allow for the entero-hepatic recycling of urea.

Despite large differences in the $\delta^{15}N$ of lean mass and keratin (hair, nail and skin) which can range from 2 to 6‰ (Sponheimer et al. 2003), the output of N associated with keratin losses is lower than 2% of the total N loss and consequently was neglected. The whole protein is considered as a unique lean mass reservoir including the collagen.

The mathematical formalism

The different reservoirs and connecting N fluxes of the model are represented in Fig. 1. The mass transfer between reservoirs is treated by first-order kinetic reactions (Lasaga 1980). The N mass conservation of a reservoir *i* matches the relation:

Table 1 List of the symbols used in the text, their description and value

Symbol in Fig. 1	Symbol in the text	Description	N (g·day^{-1})[a]	References
Fluxes				
1	**f1**	Diet intake (N_i)	$0.38 \times W^{0.74}$	This work[b]
2	**f2**	Recycling of urea under NH_3 form (A_i)	U_{hyd}	See references for U_{hyd}
3	**f3**	Urea half-synthesis ($U1_{syn}$)	$0.5 \times 0.41 \times W^{0.76}$	Assimon and Stein (1994); Chilcott et al. (1985); Cocimano and Leng (1967); Corbello Pereira et al. (2004); Forslund et al. (1998); Gibson and Hume (2002); Hume et al. (1980); Miller and Payne (1964) Morens (2002); Young et al. (2000)
4	**f4**	Urea hydrolysis (U_{hyd})	$0.16 \times W^{0.79}$	Assimon and Stein (1994); Barboza et al. (1997); Chikunya et al. (1996); Chilcott et al. (1985); Cocimano and Leng (1967); Doreau et al. (2004); Forslund et al. (1998); Gibson and Hume (2002); Hume et al. (1980); Miller and Payne (1964); Morens (2002); Peyraud et al. (1997); Young et al. (2000)
5	**f5**	Amino acids absorption (AA_i)	$0.32 \times W^{0.77}$	This work[b]
6	**f6**	Urea half-synthesis ($U2_{syn}$)	$U1_{syn}$	See references for $U1_{syn}$
7	**f7**	NH3 residue from proteins degradation (A_{res})	$0.07 \times W^{0.66}$	This work[b]
8	**f8**	Proteins synthesis (P_{syn})	$2.60 \times W^{0.72}$	Singer (2003); Waterlow (1984); White et al. (1988)
9	**f9**	Protein degradation into amino acids (P_{deg})	*Deduced*	
10	**f10**	Ileal desquamation (Il_{deg})	*Deduced*	
11	**f11**	Fecal loss (P_{exc})	*Deduced*	
12	**f12**	Urea excretion (U_{exc})	*Deduced*	
13	**f13**	NH3 excretion (A_{exc})	*Deduced*	
			N (g)	
Reservoirs				
Box 1	*b1*	Assimilated amino acids	N_i	
Box 2	*b2*	Circulating amino acids (AA)	LM×0.01	Young et al. (1992)
Box 3	*b3*	Glutamine (Gln)	AA×0.27	Volpi et al. (1999)
Box 4	*b4*	Lean mass (LM)	$22.5 \times W^{1}$	–[c]
Box 5	*b5*	Urea	Gln×0.5	–[d]
Box 6	*b6*	Urine		
Box 7	*b7*	Feces		
Fractionation factors				
	$\Delta^{15}N_{lm-d}$	$\delta^{15}N$ difference between lean mass and diet		
	$^{15}\alpha_{DA}$	Fractionation factor during the deamination of a protein into amino acids[e] (see Table 2 for the values)		
	$^{15}\alpha_{TA}$	Fractionation factor during the transamination of a protein into amino acids[e] (see Table 2 for the values)		
	$^{15}\alpha_{OS}$	Fractionation factor associated to the change of the oxidation state of N between a reactant and the product[e] (see Table 2 for the values)		

[a] Body mass (W) is in kg

[b] These allometric relations are calculated for a zero N balance using $Il_{deg} = 0.04 \times W^{0.91}$ (Donkoh et al. 1994; Mariscal-Landin et al. 1995; Montagne et al. 2000; Morens 2002; Rowan et al. 1993; Tomé and Bos 2000); $P_{exc} = 0.38(U_{exc} + A_{exc})$ (Canh et al. 1997; Sponheimer et al. 2003; Tomé and Bos 2000); $A_{exc} = 0.1 \times U_{exc}$ (Canh et al. 1997; Petersen et al. 1998)

[c] Deduced from the fact that the protein content of an animal is roughly equal to 15% of its body weight and that proteins are composed of 15% of N

[d] Deduced from the stoechiometry of N for the urea synthesis reaction

[e] The conversion in the α form is given by the relationship: $10^3 \ln(\alpha) = \Delta$

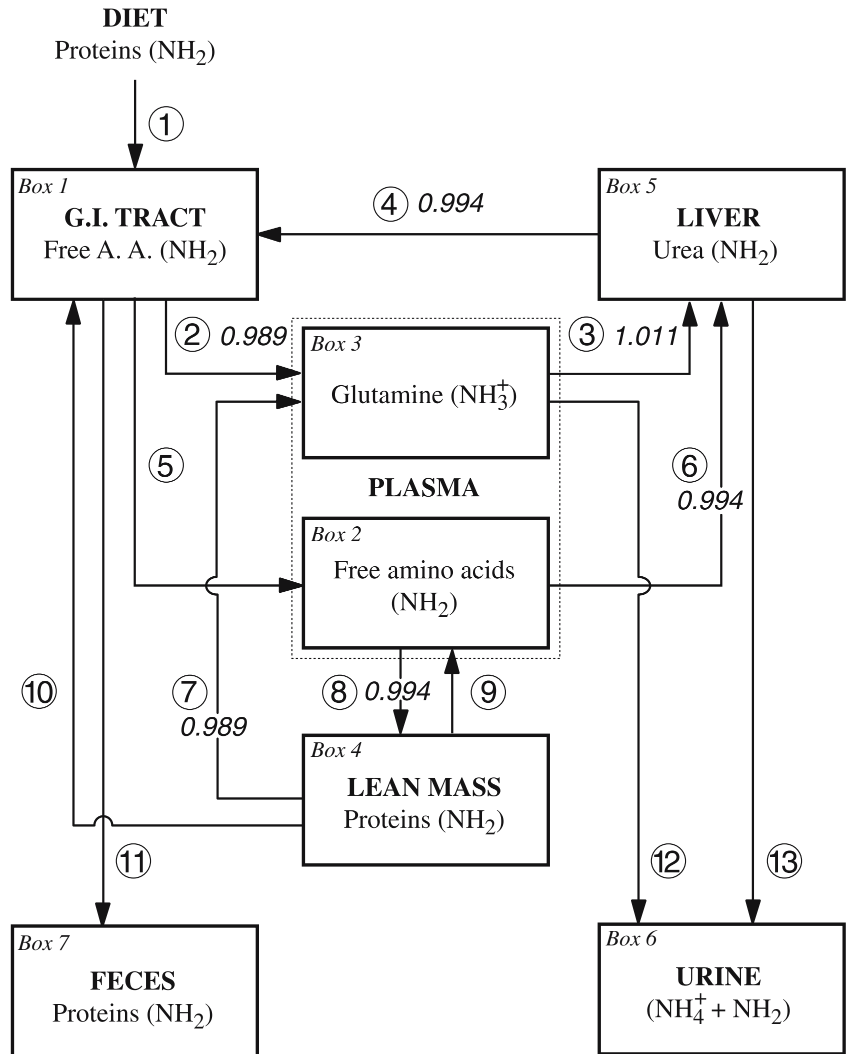

Fig. 1 Schematic diagram showing the N fluxes connecting the various reservoirs of a mammalian organism. The fluxes are described in Table 1 and associated isotopic fractionation values are given in Table 2

$$\frac{dM_i}{dt} = \sum_{j \neq i}^{j=1,n} F_{j \to i} - \sum_{j \neq i}^{j=1,n} F_{i \to j}, \quad (1)$$

where M_i is the N mass of the box i, t is the time, $F_{i \to j}$ is the N flux from box i to box j and n the number of reservoirs.

The evolution of the $^{15}N/^{14}N$ ratio of the reservoir i (R_i) is calculated following the mass conservation equation:

$$\frac{dR_i}{dt} = -\left[\sum_{j \neq i}^{j=1,n} \frac{F_{i \to j}\alpha_{i \to j}}{M_i} - \sum_{j \neq i}^{j=1,n} \frac{F_{i \to j} - F_{i \to j}}{M_i}\right] R_i + \sum_{j \neq i}^{j=1,n} \frac{F_{j \to i}\alpha_{j \to i}}{M_i} R_j, \quad (2)$$

where $\alpha_{i \to j}$ is the enrichment factor of ^{15}N due to the fractionation of N isotopes during the transfer from box i to box j. All isotopic values are expressed on the δ scale in ‰ according to the equation $\delta^{15}N = [(R_{sample}/R_{standard}) - 1] \times 1000$, where R_{sample} and $R_{standard}$ are the $^{15}N/^{14}N$ ratios of the sample and the standard (3.6765×10^{-3}), respectively.

The N content and $^{15}N/^{14}N$ ratio of each box is calculated at each time step by solving the differential equations that describe its budget (Eqs. 1, 2). Analytical solutions of a system of linear differential equations can be calculated by matrix algebra (Lasaga 1980; Albarède 1995) but the calculations and the solutions become very cumbersome when the number of reservoirs increases (e.g., Lasaga 1981). The differential system is then integrated with a numerical method by using a fourth-order Runge–Kutta algorithm (Press et al. 1992). The initial value of the $\delta^{15}N$ of each box is arbitrary set to 0‰. Note that the calculated values at steady-state do not depend on the initial conditions that have been chosen for the composition of the reservoirs.

The aim of this work is to quantify the long term (several months) $\delta^{15}N$ variations of body reservoirs assuming that the N content of the system is at steady-state for such a time-scale. This assumption is easily satisfied because several reservoirs (*b2*, *b3* and *b5*) are characterized by a N residence time, for which order of

magnitude is about 10 h. Indeed, the present model is very sensitive to small N imbalances that will dramatically shift the N concentration of the smallest reservoirs because the fluxes and the reservoirs' N masses are of the same order of magnitude.

Calibration of the $^{15}N/^{14}N$ cycle

Despite an abundant literature concerning the isotopic composition of lean mass, the values of the isotopic fractionation factors during N metabolism remain unknown. Different runs were performed to calibrate the model outputs with the well-constrained $\Delta^{15}N_{lm-d}$ value by adjusting the values of the fractionation factors.

In a first approach (Modeling A), we only considered the kinetic fractionation factors linked to transamination and deamination (Macko et al. 1986; Silfer et al. 1992). We extracted from these studies a ^{15}N isotope fractionation factor of 0.996 between the source and the product of a deamination reaction ($^{15}\alpha_{DA}$, Table 2), and a $^{15}\alpha$ value ranging from 0.990 to 0.998 for transamination ($^{15}\alpha_{TA}$, Table 2). The $\Delta^{15}N_{lm-d}$ was computed as a function of time (Fig. 2). All other body reservoirs are ^{15}N-depleted relative to diet, except the plasma free amino acids ($\delta^{15}N = 1.6‰$). This result is due to the faster incorporation of ^{14}N during protein degradation into digestible amino acids in the gastro-intestinal tract. However, the resulting overall isotopic pattern is not in accordance with the measured values.

In a second approach (Modeling B), the isotopic fractionation associated with deamination was not considered. The plasma free amino acid sub-reservoir becomes ^{15}N-enriched relative to diet ($\delta^{15}N = 6.0‰$), the other reservoirs remain unchanged in comparison to their initial $\delta^{15}N$, a pattern which is not validated by natural values.

In a third approach (Modeling C), the ^{15}N fractionation factors of transamination reactions, for which changes in the atomic neighborhood of N occur, are calculated from experimental data determined from NH_2 to NH_3^+ isotopic exchange reactions at equilibrium (Table 2). Such fractionation factors ($^{15}\alpha_{OS}$) modify the $^{15}N/^{14}N$ ratio during the reversible transformations of NH_2 to NH_3^+ during glutamine formation (**f2** and **f7**, Fig. 1), NH_3^+ to NH_2 during urea synthesis (**f3**) and NH_3^+ to NH_4^+ during the excretion of free ammonia in the urine (**f12**). Figure 3 shows the calculated $\Delta^{15}N_{lm-d}$ for a set of different $^{15}\alpha_{OS}$ and $^{15}\alpha_{TA}$ values. The isotopic fractionation associated with **f12**, which is a small flux (e.g., 0.6 gN day^{-1} for a body weight of 70 kg), only slightly modifies the $\delta^{15}N$ value ($< 0.1‰$) of all reservoirs and was thus neglected. The $^{15}\alpha_{OS}$ equilibrium values of 1.016 ($^{15}\alpha_{NH_3^+-NH_2}$), 0.984 ($^{15}\alpha_{NH_2-NH_3^+}$) and 0.984 ($^{15}\alpha_{NH_2-NH_3^+}$) are assigned to **f3**, **f2** and **f7**, respectively. They led to a $\delta^{15}N$ increase of the lean mass between 5 and 8‰ depending on the value of $^{15}\alpha_{TA}$ (Fig. 3), which is higher than the measured $\Delta^{15}N_{lm-d}$. This discrepancy is reduced when the value of $^{15}\alpha_{OS}$ is closer to unity. The measured range of $\Delta^{15}N_{lm-d}$ (3–5‰) is thus matched for a series of $^{15}\alpha_{TA}$ and $^{15}\alpha_{OS}$ values ranging from 0.990 to 0.998 and ± 1.015 to ± 1.007, respectively (Fig. 3).

In order to test the influence of these various fractionation values on the steady-state $\delta^{15}N$ of the other reservoirs, two modeling cases were considered with the maximum (Modeling D1) and the minimum (Modeling D2) values of $^{15}\alpha_{TA}$ and $^{15}\alpha_{OS}$ that bracket the measured range of $\Delta^{15}N_{lm-d}$ (Table 2). The results obtained with these two sets of values show little variations in the steady-state $\delta^{15}N$ (1‰) of intestinal amino acids, feces, urea and urine (Fig. 4). However, $\delta^{15}N$ of plasma amino acids and glutamine at steady-state differ by about 10‰ between these two boundary values. Measurements of the natural $\delta^{15}N$ of individual amino acid are sparse and highly variable (Merritt and Hayes 1994; Macko et al. 1997; Metges and Petzke 1997) and do not allow direct comparison with our data. Several studies demonstrated

Table 2 List of the values of the fractionation factors used for calculations

Flux	Description	Modeling A	Modeling B	Modeling C	Modeling D1	Modeling D2	Assigned values
1	Diet intake (N_i)	$^{15}\alpha_{DA} = 0.996$[a]	–	–	–	–	–
2	Recycling of urea under NH_3 form (A_i)	$^{15}\alpha_{TA} = 0.998$–0.990[b]	*See Modeling A*	$^{15}\alpha_{OS} = 0.996$–0.984[c]	$^{15}\alpha_{OS} = 0.985$	$^{15}\alpha_{OS} = 0.993$	0.989
3	Urea half-synthesis ($U1_{syn}$)	$^{15}\alpha_{TA} = 0.998$–0.990[b]	*See Modeling A*	$^{15}\alpha_{OS} = 1.016$–1.004[d]	$^{15}\alpha_{OS} = 1.015$	$^{15}\alpha_{OS} = 1.007$	1.011
4	Urea hydrolysis (U_{hyd})	$^{15}\alpha_{TA} = 0.998$–0.990[b]	*See Modeling A*	*See Modeling A*	$^{15}\alpha_{TA} = 0.990$	$^{15}\alpha_{TA} = 0.998$	0.994
6	Urea half-synthesis ($U2_{syn}$)	$^{15}\alpha_{TA} = 0.998$–0.990[b]	*See Modeling A*	*See Modeling A*	$^{15}\alpha_{TA} = 0.990$	$^{15}\alpha_{TA} = 0.998$	0.994
7	NH_3 residue from proteins degradation (A_{res})	$^{15}\alpha_{DA} = 0.996$[a]	–	$^{15}\alpha_{OS} = 0.996$–0.984[c]	$^{15}\alpha_{OS} = 0.985$	$^{15}\alpha_{OS} = 0.993$	0.989
8	Proteins synthesis (P_{syn})	$^{15}\alpha_{TA} = 0.998$–0.990[b]	*See Modeling A*	*See Modeling A*	$^{15}\alpha_{TA} = 0.990$	$^{15}\alpha_{TA} = 0.998$	0.994
9	Protein degradation into amino- acids (P_{deg})	$^{15}\alpha_{DA} = 0.996$[a]	–	–	–	–	–

[a]Data calculated from Silfer et al. (1992) with the values given in Table 3 for a temperature of 100°C
[b]Data calculated from Macko et al. (1986) with the values given in Table 1
[c]Data calculated from Hermes et al. (1985) given in Table 4 of Rishavy and Cleland (1999) as $\alpha_{NH2-NH3}/\alpha_{NH2-NH3+}$
[d]Data calculated from Hermes et al. (1985) given in Table 4 of Rishavy and Cleland (1999) as $\alpha_{NH2-NH3+}/\alpha_{NH2-NH3}$

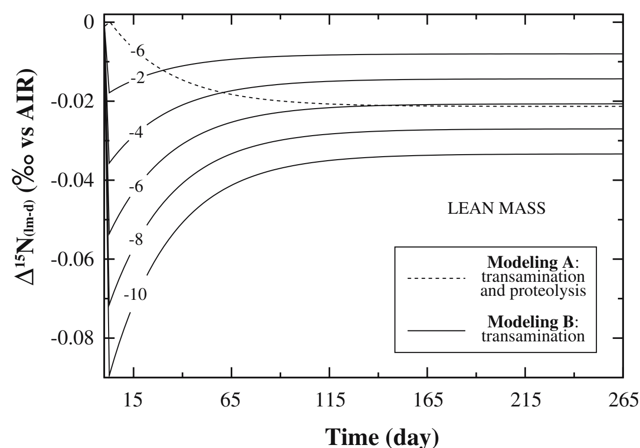

Fig. 2 Evolution over 9 months of the computed ^{15}N-enrichment between lean mass and diet using fractionation factors associated with transamination and proteolysis (see Table 2 for details)

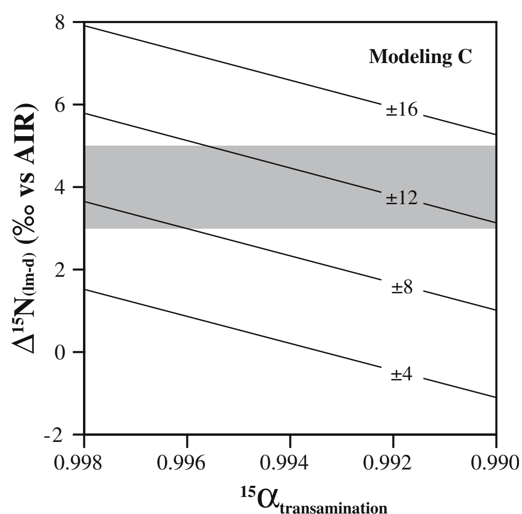

Fig. 3 Variation of the ^{15}N-enrichment between lean mass and diet as a function of $^{15}\alpha_{TA}$ and $^{15}\alpha_{OS}$. The values of $^{15}\alpha_{OS}$ are given as $\Delta^{15}N_{OS}$. The *shaded area* covers the accepted range of ^{15}N-enrichment between lean mass and diet (3–5‰)

that the δ^{15}N of plasma is always close to the δ^{15}N of lean mass (e.g., Hobson et al. 1996; Ambrose 2000; Roth and Hobson 2000). No direct δ^{15}N measurements were reported for glutamine because separating it from glutamate by gas chromatography remains difficult (e.g., Metges and Petzke 1997).

This calibration procedure finally led to assign a value of 0.994 for $^{15}\alpha_{TA}$, that is the mean value for the ^{15}N-depletion range during transamination (Macko et al.

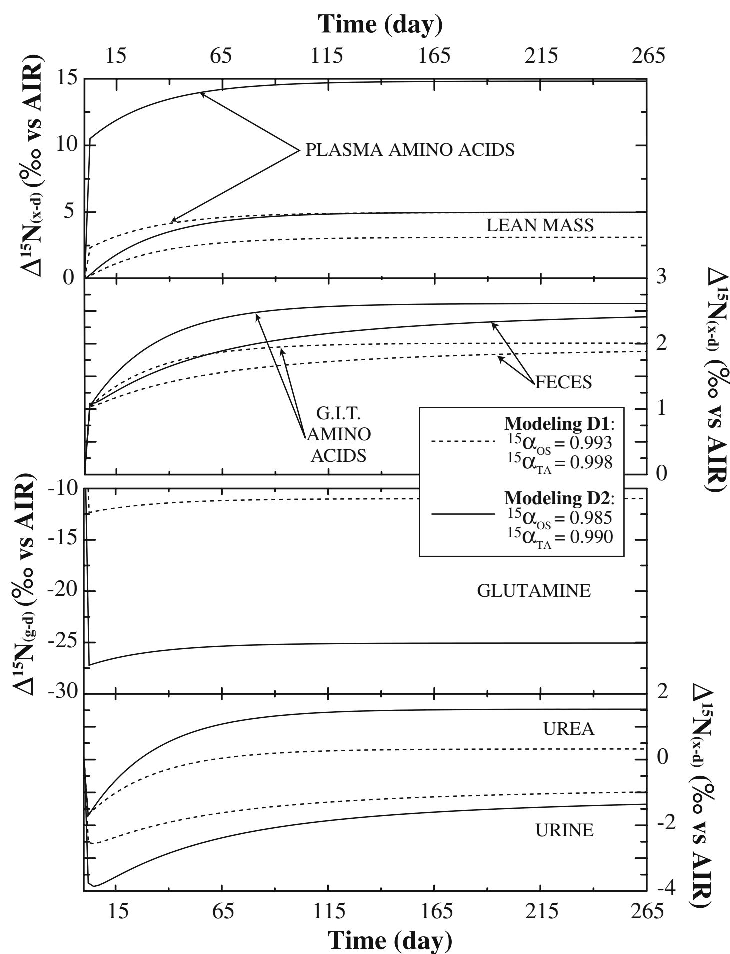

Fig. 4 Evolution over 9 months of the computed ^{15}N-enrichment between the different body reservoirs and diet

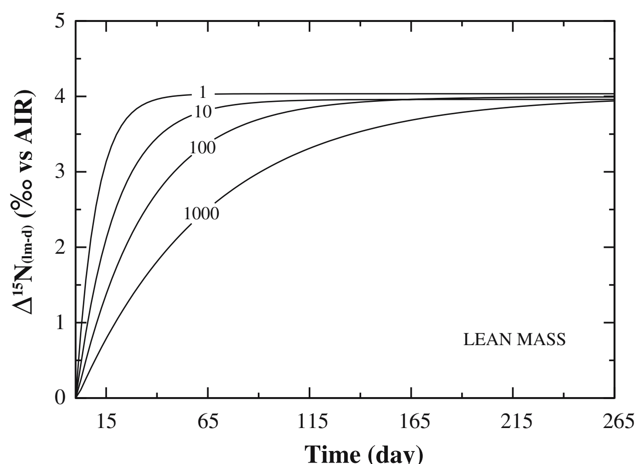

Fig. 5 Evolution over 9 months of the computed ^{15}N-enrichment between lean mass and diet for different body weights

1986). In order to set the $\Delta^{15}N_{lm-d}$ close to 4‰, $^{15}\alpha_{OS}$ values of 1.011, 0.989 and 0.989 were assigned to **f3**, **f2**, and **f7**, respectively (Table 2). By using these fractionation factors, at steady-state, we obtained δ^{15}N values of −18.0‰ for the glutamine, 9.9‰ for the plasma amino acids, 2.3‰ for the intestinal amino acids, 4.1‰ for the lean mass, 0.9‰ for the urea, −1.1‰ for the urine, and 2.2‰ for the feces.

Results and discussion

The modeling was performed to determine the influence of the different body masses on the time required for the different reservoirs to reach isotopic steady-state. The steady-state δ^{15}N of lean mass (4‰) remains unchanged from 1 to 10^3 kg (Fig. 5). The time required for lean mass to reach δ^{15}N steady-state increases as a function of body mass from 1 month (1 kg) to more than 7 months (10^3 kg). These results add time-dependent constraints for performing experimental investigations.

Published experimental δ^{15}N between lean mass, plasma, excreta, and diet for mammals (Table 3) are compared to the results of our ^{15}N/^{14}N box-modeling. The ^{14}N fluxes are scaled according to the estimated body mass of a given mamm al species. The models are computed according to the duration of the experiment along with the reported diet δ^{15}N and the N intake (g·day^{-1}) (Table 3). The computed δ^{15}N values of feces and urine of llamas and horses (Fig. 6a) are compared to the experimental values obtained by Sponheimer et al. (2003). The differences between the δ^{15}N of urine and feces for each experiment are relatively well reproduced by the model (Fig. 6a). However, the computed isotopic compositions are systematically ^{15}N-depleted by 3‰ in comparison to the measured values. This offset is likely the result of short-lived experiments (about 1 month, see Table 3) leading to underestimate the $\Delta^{15}N_{lm-d}$ value because body masses of about 100 kg cannot reach steady-state isotopic compositions (Fig. 5). These results highlight the need of an adequation between the duration of experiments and the body mass of studied mammals.

The predicted δ^{15}N values of plasma amino acids (Fig. 6b) are from +2 to +8‰ ^{15}N-enriched relatively to the measured values. The difference between predicted and measured δ^{15}N values of amino acids decreases as a function of the increasing value of $^{15}\alpha_{TA}$. This pattern is clearly illustrated by the decrease in the predicted values of seal δ^{15}N plasma amino acids (Hobson et al. 1996) from 23 to 16‰ for values of $^{15}\alpha_{TA}$ varying from 0.996 to 1 (Fig. 6b).

The concomitant measurements of lean mass and plasma amino acid δ^{15}N of animals fed with an isotopically controlled diet are sparse (Hobson et al. 1996; Roth and Hobson 2000) and do not allow any conclusion about the potential systematic difference between the isotopic compositions of these two reservoirs. The documented Δ^{15}N between diet and plasma of mammals gives an average value of 3.8±1.1‰ (Robbins et al. 2005) that is comparable to the accepted range of ^{15}N-enrichment between diet and lean mass. These results

Table 3 List of the parameters used in the calculations for Fig. 6a, b

Animal	Weight (kg)[a]	Feeding duration (day)	Diet δ^{15}N (‰ air)	N intake (g·day^{-1})	Body δ^{15}N (‰ air)[b]	References
Rat	0.2	>90	3.3–7.8	1.5–15.1[c]	6.2–11.0 (LM)	Ambrose (2000)
Rat	0.2	Not reported	4.6–5.8	1.5[c]	7.6–8.6 (LM)	DeNiro and Epstein (1981)
Rat	0.2	Not reported	6.1	1.5[c]	8.4–9.6 (LM)	Minagawa and Wada (1984)
Fox	10	210	4.9	2.1[d]	8.5 (LM); 9.1 (P)	Roth and Hobson (2000)
Bear	250	40	2.3–15.6	22[d]	6.5–20.5 (P)	Hilderbrand et al. (1996)
Seal	100	>720	13.0	11.4[d]	15.6 (LM); 14.7 (P)	Hobson et al. (1996)
Llama	130	>26	0.4–5.8	18.4–53.4	2.9–9.2 (F); −0.2–3.9 (U)	Sponheimer et al. (2003)
Horse	500	>26	0.4–5.8	155.3–309.4	3.0–9.1 (F); 0.3–3.8 (U)	Sponheimer et al. (2003)

[a]Taken from the URL:http://animaldiversity.ummz.umich.edu/site/index.html
[b]LM, P, F, and U denote for lean mass, plasma, feces, and urine, respectively
[c]Calculated from the relation $N_i=0.21\times\%$Prot.$+0.48$ (Morens 2002)
[d]Calculated from the N balance for the given the body mass (flux 1 of Table 1)

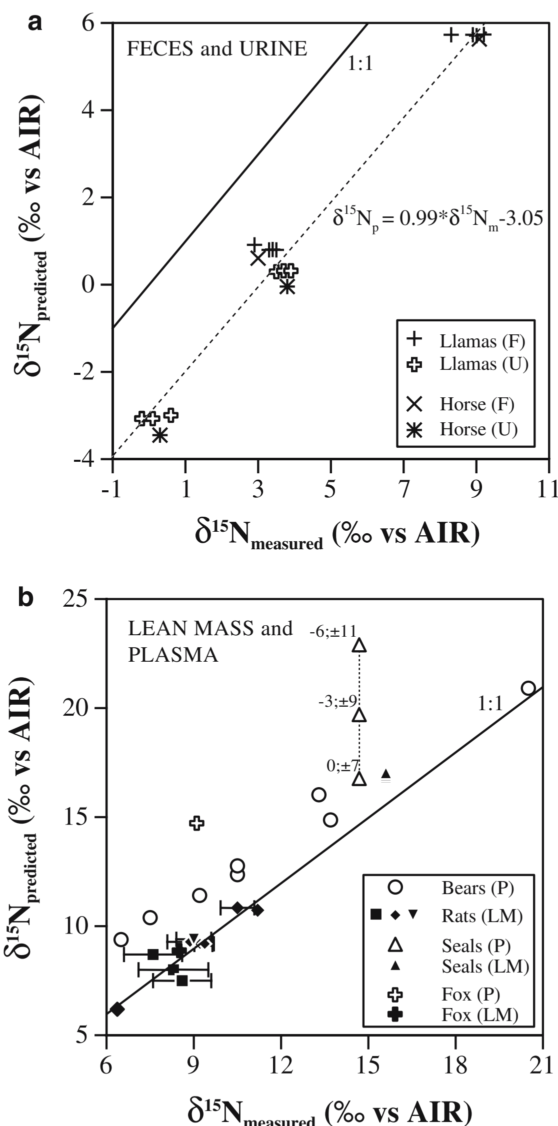

Fig. 6 **a** Computed δ^{15}N values of feces (F) and urine (U) reported against the measured values from Sponheimer et al. (2003). **b** Computed δ^{15}N values of lean mass (LM) and plasma amino acids (P) reported against the measured values. Data from: *circles*, Hilderbrand et al. (1996); *square*, DeNiro and Epstein (1981); *losange*, Ambrose (2000); *lower triangle*, Minagawa and Wada (1984); *upper triangle*, Hobson et al. (1996); *cross*, Roth and Hobson (2000). The numbers associated with the *upper triangles* represent the $\Delta^{15}N_{TA}$ and $\Delta^{15}N_{OS}$ values

suggest that the ^{15}N kinetic fractionation operating during transamination is of minor importance in the N isotope cycle of mammals. The small offset observed between calculated and measured δ^{15}N of lean mass results from the uncertainties related to the knowledge of animal masses and their N intake during the experiments.

Figure 7 illustrates the variation of the steady-state δ^{15}N values of the lean mass for a human of 70 kg fed with a N intake ranging from 4.9 to 14.9 g·day^{-1} (the calculated zero N balance equals 8.9 g·day^{-1}). A negative (respectively positive) N balance is responsible for a

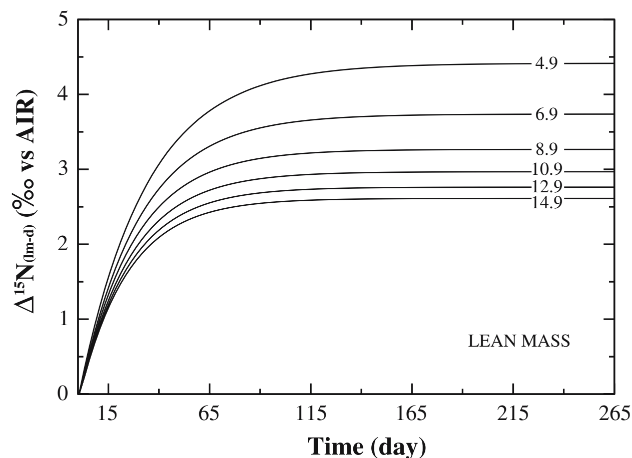

Fig. 7 Evolution over 9 months of the computed ^{15}N-enrichment between lean mass and diet for different N intakes

^{15}N-enrichment (respectively depletion) of inner reservoirs (Fig. 7). A review of the literature shows that a decrease in Δ^{15}N between diet and plasma amino acids is strongly related to an increase in the N intake (Robbins et al. 2005). Indeed, smaller is the N intake, higher is the N isotope composition of the gastro-intestinal tract as a result of the predominance of urea hydrolysis and ileal desquamation fluxes, both being ^{15}N-enriched relative to the diet.

The above result also opens new perspectives to interpret the relationship between the increase of mammal δ^{15}N with aridity (e.g., Heaton et al. 1986; Cormie and Schwarcz 1996; Gröcke et al. 1997). It is commonly postulated that during water-stress conditions, decrease in both urine and urea excretions lead to an increase in the storage of body water (e.g., Ambrose 2000). Macropod data from Gröcke et al. (1997) are compared to the modeled ^{15}N-enrichments produced by variable turnover of the urea cycle. The N cycle is slightly modified as the excess of hydrolyzed urea is incorporated into the plasma amino acids pool. There is a satisfying agreement between the observed (2.7–13.3‰) and predicted (4–11‰) ranges of lean mass δ^{15}N for N intake of 1.25 g·day^{-1} (Fig. 8). One rarely mentioned implication of the recycling of urea (Cormie and Schwarcz 1996) is the reduction of the required N intake to achieve a zero N balance. The decrease in N intake from 1.25 to 0.9 g·day^{-1} causes an increase in the ^{15}N-enrichment of about 2 and 4‰ for 60 and 100% of recycled urea, respectively (Fig. 8). Therefore, we propose that the observed increase in δ^{15}N of mammals with aridity is more likely a combined effect of water recycling and reduction of the N intake. However, much experimental work is needed to understand whether this ^{15}N-enrichment is linked to higher urea recycling during water-stress period or to the natural δ^{15}N increase in soils and plants with aridity (Amundson et al. 2003).

We also attempted to better understand the physiology of extinct cave bears (*Ursus spelaeus*). Cave bears, like other modern bears, have an unusual metabolism

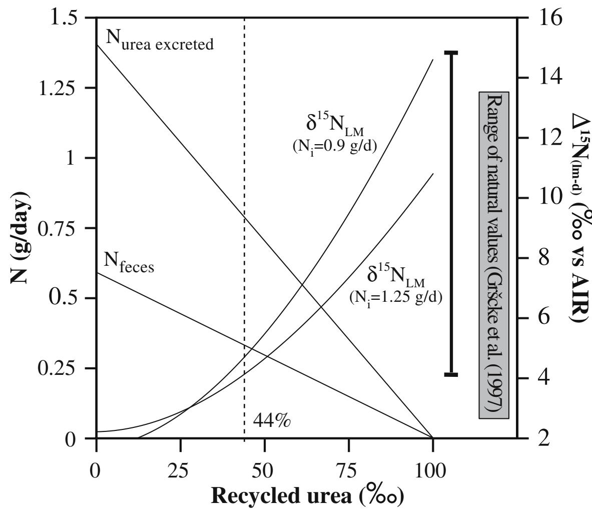

Fig. 8 Variations in the fluxes of excreta (feces and urea) and ^{15}N-enrichment between lean mass and diet as a function of the percentage of recycled urea in macropods (W = 5 kg). The *vertical dashed line* illustrates the basal percentage of recycled urea (44%). The *bold vertical bar* illustrates the computed ^{15}N-enrichment of recycled urea that ranges from 44 to 100%. The range of δ^{15}N values measured for macropods by Gröcke et al. (1997) is reported for comparison

during dormancy: they are able to maintain their basal body temperature without food consumption, defecation or urination (Barboza et al. 1997; Farley and Robbins 1995). Bocherens et al. (1994) and Nelson et al. (1998) suggested that marked δ^{15}N differences among tissues (bone, dentine of deciduous and permanent teeth) of cave bears result from the N metabolism during dormancy. An annual hyperphagia–dormancy cycle is simulated for a bear weighting 50 kg, with a N intake of 75 g·day^{-1} during hyperphagia (Barboza et al. 1997). During dormancy, the diet, gastro-intestinal tract, feces and urine reservoirs are removed from the N isotope cycle. The hydrolysis of urea is separated into two fluxes,

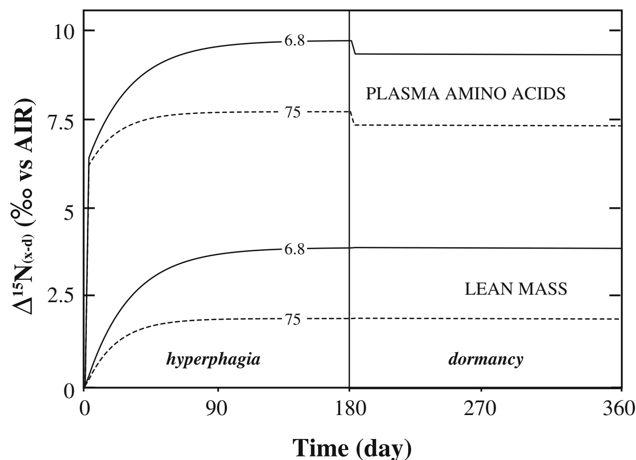

Fig. 9 Evolution over 1 year of the computed ^{15}N-enrichment between lean mass, plasma amino acids, and diet for a bear fed with different N intakes

the first one is connected to the free amino acid sub-reservoir (**f5′** = 6.4 gN day^{-1}) and the second one to the glutamine sub-reservoir (**f2′** = **f3** + **f6** - **f5′** = 1.6 gN day^{-1}). No δ^{15}N difference is calculated between hyperphagia and dormancy for the lean mass and amino acid reservoirs (Fig. 9) because the N cycle is at steady-state after 6 months of hyperphagia and behaves as a closed system during dormancy. Thus, the odd δ^{15}N values observed for cave bears cannot be explained by the dormancy phenomenon alone. The only way to modify the steady-state of the N isotope cycle during dormancy is to add a ^{14}N sink, which can be achieved by gestation or suckling. Assuming that this sink is linked to the free amino acid sub-reservoir, it would have a δ^{15}N value close to this latter. This should explain why the offspring of cave bears has high δ^{15}N values (7–5‰, Nelson et al. 1998) while adults are generally characterized by lower values (e.g., Bocherens et al. 1994). It is noteworthy that the δ^{15}N of plasma amino acids of neonates is correlated to the δ^{15}N of the mother's diet for non-hibernating mammals (Jenkins et al. 2001).

In the case of δ^{15}N values of cave bears that are lower than those of coeval herbivores (Bocherens et al. 1994), the low δ^{15}N values of cave bears could be explained by hyperphagia considering that a positive N balance is related to a decrease in the steady-state δ^{15}N values (Fig. 7). This case is illustrated in Fig. 9 where the δ^{15}N values of lean mass equal 1.9 and 3.9‰ for a N intake of 75 and 6.8 g·day^{-1}, respectively.

Conclusions

The aim of this study was to perform a time-dependent box-modeling of nitrogen isotope ratios of mammals that takes into account the isotopic fractionations associated with speciation changes during the NH_3 cycle. Results of modeling show that a combination of kinetic isotope fractionations during the N transfer between amines and equilibrium fractionations related to the reversible conversion of N-amine into ammonia is required to account for the well-established ≈4‰ ^{15}N-enrichment of body proteins relative to the diet. This isotopic enrichment observed in proteins is due to the partial recycling of ^{15}N-enriched urea and the urinary excretion of a fraction of the strongly ^{15}N-depleted ammonia reservoir. For a given body mass and diet δ^{15}N, the isotopic compositions are mainly controlled by the N intake.

The application of our model to case studies suggests, for example, that the observed increase of δ^{15}N of mammals with aridity is a combined effect of water recycling and reduction of the N intake. The intriguing low δ^{15}N values of cave bears relative to coeval herbivores are interpreted as the result of hyperphagia instead of dormancy.

We stress that more experimental works are needed to understand well the natural variability of the ^{15}N-enrichment between an animal and its diet. Future

experiments should be designed to adapt the duration of experiments to the body mass of studied mammals in order to reach steady-state nitrogen isotope compositions.

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