Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt

Lehrstuhl für Grünlandlehre

Carbon and nitrogen isotope turnover in animals and animal products

Alexander Braun

Vollständiger Abdruck der von der Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt der Technischen Universität München zur Erlangung des akademischen Grades eines

Doktors der Naturwissenschaften

genehmigten Dissertation.

Vorsitzender: Univ.-Prof. Dr. J. Schnyder

Prüfer der Dissertation:

1. apl. Prof. Dr. K. F. Auerswald
2. Univ.-Prof. Dr. J. P. Geist

Die Dissertation wurde am 06.02.2014 bei der Technischen Universität München eingereicht und durch die Fakultät Wissenschaftszentrum Weihenstephan für Ernährung, Landnutzung und Umwelt am 09.04.2014 angenommen.

„Der Natur gegenüberzustehen

und seinen Scharfsinn an ihren Rätseln zu erproben,

gibt dem Leben einen ungeahnten Inhalt.“

Alfred Wegener (1880 – 1930)

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Summary

Knowledge on the isotopic turnover is used for diet reconstruction in animal ecology, paleontology, forensics, and food authentication. The isotopic turnover determines the time period during which the isotopic composition of an organismic material represents its dietary input. This study (i) quantified the isotopic turnover by compartmental modeling of a systematic isotopic-switch experiment and validated the turnover information at different (dietary) conditions by forward modelling, (ii) applied inverse modelling of natural isotopic fluctuations to quantify isotopic turnover without systematic switch experiments, (iii) analyzed the effect of dietary protein content on isotopic turnover by piecewise linear/non-linear modeling and (iv) analyzed the compound specific isotopic turnover of amino acids and their specific dietary information.

Isotopic carbon and nitrogen compositions of the diet and of 28 organismic materials were obtained from three isotopic-switch experiments. In particular, whole milk, casein, lactose, milk fat, feces and hair were sampled from the cow (*Bos taurus*) and urine, feces, plasma, liver, kidney, lung, spleen, brain, heart, muscle and twelve muscular amino acids were sampled from the rat (*Rattus norvegicus*). The masses of whole animals and of organs were weighed during the experiments to correct the isotopic turnover in case of growth. A delay between ingestion and first manifestation of dietary isotopes in organismic materials was considered for statistical analysis.

Overall, the delay was ~12 h and the turnover was best represented by one-pool models, except for whole-milk, which was best represented by a two-pool model. In particular, it was found that (i) the two-pool model of milk represented the turnover of its components and that the isotopic turnover information obtained from a systematic isotopic-switch experiment allowed the prediction of isotopic fluctuations from different (dietary) conditions, (ii) isotopic turnover could be quantified by inverse modeling of natural isotopic fluctuations, (iii) an increase in dietary protein content increased isotopic turnover in all organs of the rat and (iv) amino acids of rat's muscle had a consistent nitrogen isotope turnover, but different nitrogen isotope compositions that carried different dietary

information depending on the amino acids' abilities of *de novo* synthesis and transamination.

The results suggest that (i) delays should be considered for isotopic turnover estimation, (ii) different tissues within an animal comprise half-lives covering about two orders of magnitude from hours to months, (iii) carbon and nitrogen half-lives are similar within the same material, and are similar for (artificial) single-switch and (natural) multiple-switch diets. Thus, different materials within one organism provide a rather reliable isotopic clock for diet reconstruction, if the dietary protein content of the organism remained rather constant. The synopsis of isotopic turnover beyond species barriers revealed that the delay was rather independent of digestive systems (monogastric vs polygastric) and that the half-lives of organs of small model organisms, like rats, can be used to predict the half-lives of organs of bigger organisms, like cows.

Zusammenfassung

Die Analyse von stabilen Isotopen in tierischen Geweben erlaubt Rückschlüsse auf die Nahrungsaufnahme von Tieren. Dazu benötigt man Informationen über den gewebespezifischen Umsatz von stabilen Isotopen. Die Ziele dieser Studie waren, (i) mittels kompartimenteller Modellierung den isotopischen Umsatz in Milch und Milchkomponenten an Hand eines Isotopieumstellungsversuches zu quantifizieren, um anschließend mittels Vorwärtsmodellierung zu validieren, ob die Umsatzinformation auch unter natürlichen Bedingungen (~ isotopische Fluktuationen) gilt, (ii) den isotopischen Umsatz unter natürlichen isotopischen Fluktuationen mittels Rückwärtsmodellierung zu bestimmen, (iii) den Effekt des Proteingehaltes der Nahrung auf den isotopsichen Umsatz zu testen, und (iv) den Umsatz von N-Isotopen für einzelne Aminosäuren zu messen und die Aminosäure-spezifische isotopische N-Zusammensetzung in tierischen Geweben zu erklären.

Dafür wurden die isotopischen Zusammensetzungen von Milch, Laktose, Kasein, Milchfett, Kot und Haare der Kuh (*Bos taurus*) sowie von Urin, Kot, Blutplasma, Leber, Niere, Lunge, Milz, Gehirn, Herz, Muskel und von zwölf Aminosäuren des Muskels der Ratte (*Rattus norvegicus*) in drei Isotopieumstellungsversuchen gemessen. Das Gewicht der Tiere und der Organe wurde während der Experimente gemessen, um den isotopischen Umsatz in Falle von Wachstum zu korrigieren. Eine zeitliche Verzögerung zwischen Nahrungsaufnahme und Festsetzung der isotopischen Komposition der Nahrung im tierischen Gewebe wurde berücksichtigt.

Die zeitliche Verzögerung war über alle Gewebe hinweg ca. 12 h. Der folgende isotopische Umsatz wurde am besten von ein-pool-Modellen repräsentiert; ausschließlich bei der Milch wurde ein zwei-pool-Modell benötigt. Im Speziellen wurde festgestellt, (i) dass das zwei-pool-Modell der Milch den individuellen Umsatz der Milchkomponenten repräsentierte. Außerdem konnte die Information über den isotopischen Umsatz aus systematischen Isotopieumstellungsversuchen die natürlichen isotopischen Fluktuationen bei natürlicher Ernährung erklären. (ii) Der isotopische Umsatz konnte mittels Rückwärtsmodellierung auch ohne Isotopieumstellungsversuche quantifiziert werden. (iii)

Eine Erhöhung des Proteingehaltes der Nahrung bewirkte eine Beschleunigung des isotopischen Umsatzes in tierischen Geweben und (iv) der Stickstoff verschiedener Aminosäuren hatte zwar einen ähnlichen isotopischen Umsatz, enthielt jedoch unterschiedliche Informationen über die Nahrung.

Die Ergebnisse legen nahe, dass (i) zeitliche Verzögerungen bei der Bestimmung des isotopischen Umsatzes berücksichtigt werden sollten, (ii) dass die Halbwertszeiten von verschiedenen Gewebe desselben Tieres eine Spanne von Faktor 100 aufweisen, (iii) dass die Halbwertszeiten von Kohlenstoff- und Stickstoffumsatz innerhalb eines Gewebes ähnlich sind, und dass die Halbwertszeiten sich zwischen (künstlichen) Isotopieumstellungsversuchen und (natürlichen) mehrfachen Isotopieumstellungen nicht unterscheiden. Verschiedene Gewebe desselben Tieres bieten deshalb die Grundlage für eine robuste isotopische Uhr für die Nahrungsrekonstruktion, wenn der Nahrungsproteingehalt des Tieres nicht zu sehr schwankt. Eine Zusammenschau der Ergebnisse der verschiedenen Organismen offenbarte, dass die zeitliche Verzögerung unabhängig vom Verdauungssystem war (Monogaster vs Polygaster). Ebenso erlaubte die isotopische Umsatzinformation von Organen von kleinen Modellorganismen, wie Ratten, die Vorhersage der isotopischen Umsatzinformation von Organen von weit größeren Organismen, wie Kühen.

General Introduction

Turnover in the biological sense is the continuous process of loss and replacement of a constituent of a living system (Merriam-Webster's Collegiate Dictionary, 2005). Due to the variety of living systems – hierarchically expanding from cells to the biosphere (Lobo, 2008) – as well as due to the variety of their constituents, many different biological turnovers exist and have been investigated, e.g. the turnover of proteins in organs (Biolo *et al*., 1995), the turnover of cells in organisms (Spalding *et al*., 2008) and the turnover of atoms in the biosphere (Barrett, 2002).

The fields of animal ecology, paleontology, forensics and food authentication focus on the turnover of stable isotopes in organismic materials, mainly in organs and excreta (Martinez del Rio *et al*., 2009). In contrast to organs, excreta like feces, hair and milk remain metabolically inactive once synthesized and do not turnover, but their analysis provides an "apparent turnover" representing the isotopic turnover of their constituents in the body. Since isotopic turnover includes endogenous recycling of elements, isotopic turnover times – denoted in half-life – are longer compared to the turnover times of most other turnovers (e.g. protein turnover) that rather represent the pure synthesis times (Garlick *et al*., 1994).

Isotopic half-lives provide researchers with the time window through which they can perceive information when analyzing the isotopic composition of a specific material (Martinez del Rio *et al*., 2009). This is frequently used for diet reconstruction, providing time- and space-integrated insights into trophic relationships (Layman *et al*., 2012), natural diet switches (Cerling *et al*., 1999), geographical origin and migration patterns (Trueman *et al*., 2012). Also, the quantification of isotopic diet-tissue shifts (also called trophic shifts) requires consideration of the turnover (Auerswald *et al*., 2010; Codron *et al*., 2012).

The isotopic turnover is quantified by switching the isotopic composition of a specific element of the diet and modeling the delayed exponential reaction progress of the isotopic composition in an organismic material (Fig. 1, Sponheimer *et al*., 2006; Lecomte *et al*., 2011). The delay between isotopic switch and reaction progress is likely caused by

mastication, digestive transit, passing the gastro-intestinal barrier, transportation in the systemic circulation and delayed synthesis of new components from precursors (Cerling *et al*., 2007). Delays have been found in birds and sheep ranging from three days to two weeks (Cerling *et al*., 2007; Zazzo *et al*., 2008). Neglecting the delay can interfere the half-life estimation, in particular it may lead to an overestimation of half-lives (Cerling *et al*., 2007).

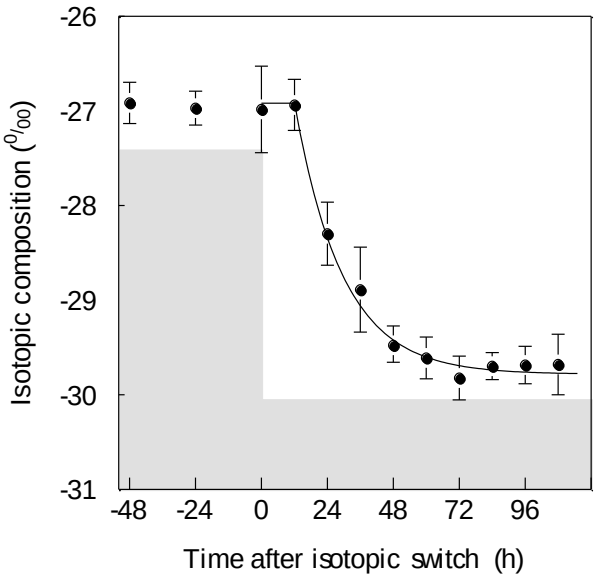

Figure 1: The delayed reaction progress in isotopic composition of an organismic material after switching the isotopic composition of the diet. A (one-pool) model that includes delay and reaction progress is indicated by a solid line. The isotopic composition of the diet is shaded in grey.

The half-life can be derived from the following reaction progress by fitting a mono-exponential function (i.e. one-pool model). Isotopic half-lives range from 1 d to > 100 d, differing between organisms (Bahar *et al*., 2009; Kurle 2009), between organs of the same organism (Miller *et al*., 2008) and between chemical compounds of the same organ (Martineau *et al*., 1985). By fitting multi-exponential functions, i.e. compartmental or multi-pool modelling, also number, sizes and interactions of elemental pools and sources that contribute to an organismic material can be analysed (Lehmeier *et al*., 2008). However, compartmental modelling is currently limited by a lack of understanding how the reaction progress of multiple pool systems can be disentangled and interpreted.

According to most metabolic processes, isotopic turnover and hence half-lives vary with physiological (e.g. aging, Lecomte *et al*., 2011) and environmental factors (e.g. temperature, Witting *et al*., 2004). Yet, there is a lack of knowledge on dietary effects on the isotopic carbon and nitrogen turnover in different organismic materials. If there was a dietary effect, the validity of half-lives in diet reconstruction studies may be questioned for two reasons. (i) The isotopic switch is usually achieved by a diet switch, which is usually associated with changes in its chemical and morphological properties. (ii) Half-lives are quantified in diet-switch experiments but are applied to more natural dietary conditions. While the first caveat could be overcome by an "isotopic-switch, but constant-diet experiment", the second caveat could be overcome by a method that quantifies isotopic turnover at more natural conditions (e.g. using natural isotopic fluctuation instead of a systematic switch). Further it is unknown, how isotopic turnover differs between specific compounds (e.g. amino acids) and its composite materials (e.g. bulk muscle). Such compound specific isotopic information might give new perspectives into isotopic diet reconstruction.

The present work investigated the isotopic turnover of carbon and nitrogen in several organismic materials of differing biological complexity (Table 1). The materials were sampled from different organisms, namely *Bos taurus* and *Rattus norvegicus* that differ substantially in size and in digestive systems. Rats are rather small and monogastric, whereas cows are rather large and polygastric. Although, the type of digestive system has a considerable effect on metabolic processes (Krömker, 2006), effects of digestive systems on the isotopic turnover are unknown. The rumen of polygastric systems can be considered a bioreactor that allows even the digestion of structural carbohydrates that are indigestible fibers for monogastric organisms, e.g. cellulose and hemicellulose. Such an additional carbon pool could affect the carbon turnover of organs and excreta. An additional effect might arise from the higher nutrient resorption potential in polygastric systems (Loeffler & Gäbel, 2013). Since body mass was also found to affect the rate of isotopic incorporation (at least within one species, Martinez del Rio *et al*., 2009), differences in isotopic turnover between rat and cow can be expected.

Table 1: The investigated organismic materials according to their hierarchical biological complexity and their animal species.

Decreasing biological complexity →	**Investigated materials**
Organ	plasma[2], kidney[2], muscle[2], liver[2], heart[2], lung[2], spleen[2], brain[2]
Excreta	hair[1], feces[12], urine[2], milk[1]
Compound class	milk fat[1], milk casein[1]
Molecular compounds	amino acids[2], milk lactose[1]

[1] in *Bos taurus*
[2] in *Rattus norvegicus*

In particular, (i) it was tested whether isotopic turnover information obtained from a systematic isotopic-switch experiment is valid for practical feeding conditions that include naturally fluctuating isotopic compositions in diet inputs and in metabolic outputs (milk, milk components and feces). Further, it was investigated whether compartmental modelling of a composite material like milk (consisting of the milk components milk fat, casein and lactose) allows drawing conclusions on the individual milk components. (ii) It was evaluated whether inverse modelling of natural fluctuations of isotopic composition in the diet and in metabolic outputs allows quantifying the isotopic turnover without applying a systematic isotopic-switch experiment. (iii) The effect of dietary protein content on the isotopic carbon and nitrogen turnover was tested in ten rat materials. To this end, a statistical approach that combines the estimation of delay and half-life by fitting linear/non-linear models was developed. Additionally, the range of carbon and nitrogen half-lives within one organism should be elucidated and compared to the range of isotopic turnover found in literature. (iv) Finally, the compound specific isotopic turnover of amino acids and their specific dietary information were analyzed.

General methods

Ethics statement

The study was conducted with cows (*Bos taurus*) and Sprague-Dawley rats (*Rattus norvegicus*). The experiment with cows involved only typical agricultural practices of animal keeping. Thus, no specific regulations/laws applicable for experiments involving vertebrates had to be followed. The animals were kept and fed at the farm of the University of Applied Sciences Weihenstephan, under the authority of the government of the Bavarian administrative district Oberbayern. All animals were healthy and remained at the farm after the conclusion of the experiment. Blood was drawn by an attending veterinarian for monitoring of health and well-being. For the experiment with rats, all procedures and protocols were approved by the Animal Care and Use Committee of Technische Universität München.

Isotopic analysis

Samples (dry matter: 0.7 mg, SD 0.05 mg; liquids: 5 µl, SD 0.1µl) were transferred into tin cups and were combusted in an elemental analyzer (NA 1110, Carlo Erba, Milan, Italy) interfaced (Conflo III, Finnigan MAT, Bremen, Germany) to an isotope ratio mass spectrometer (Delta Plus, Finnigan MAT). Solid internal lab standards (SILS, protein powder) were run as a control after every tenth sample. All samples and SILS were measured against a reference gas, which was previously calibrated against secondary international standards from the International Atomic Energy Agency, Vienna, Austria (IAEA-CH6, IAEA-N1, IAEA-N2). The long-term precision for the internal lab standards was better than 0.20 ‰ for $\delta^{13}C$ values and $\delta^{15}N$ values. Isotope data are usually presented in δ notation [$\delta = (R_{sample}/R_{standard}) - 1$, with R the isotope ratio ($^{13}C/^{12}C$, $^{15}N/^{14}N$) in the sample or international standard (VPDB, AIR)]. In contrast, the fourth study, "Transamination reduces heterogeneity in isotopic nitrogen composition of amino acids in animals", expressed the isotopic nitrogen composition in atom fraction $x(^{15}N)_P$, because its

use is preferred over the use of δ values for tracer and mixing calculations (Brenna *et al*. 1997, Coplen 2011):

$$x(^{15}N)_P = n(^{15}N)/(n(^{14}N) + n(^{15}N)) \quad \text{(Eqn 1)},$$

where $n(^{14}N)$ and $n(^{15}N)$ are the amounts of isotope ^{14}N and ^{15}N of element N in sample *P*, respectively. All calculations were done in R (R Core Team, 2013) with the auxiliary package nlme (Pinheiro *et al*., 2012).

Experiment 1: Forward modeling of fluctuating dietary ^{13}C signals to validate ^{13}C turnover models of milk and milk components from a diet-switch experiment

Animals, feeding and labelling

The diet switch experiment was performed with eight Simmental cows selected from a larger herd to yield a subsample with similar age, milk production and phase of lactation. The cows were 4.2 years old (SD 0.4 yr; SD indicating the standard deviation), produced 22.6 L d^{-1} (SD 1.6 L d^{-1}) of milk and were in mid lactation (162 d in milk SD 14 d). However, the cows differed in live weight by up to 153 kg (mean 640 kg SD 56 kg, minimum 550 kg, maximum 703 kg). Each cow's weight stayed approximately constant (SD 3 %) during the experiment.

Prior to the experiment, the cows grazed for six weeks on a pasture, where grass (C_3) was the only feed source. From the start of the experiment, the cows were fed a mixed diet consisting of fresh pasture grass supplemented with maize meal (C_4) for eight weeks (this period was termed the 'isotopic equilibration period'). The pasture grass was fed *ad libitum*. The maize meal was given at a constant rate (dry matter: 1.7 kg cow^{-1} d^{-1}). The digestibility of the maize meal and pasture grass was high and near identical (80% and 77%, respectively, Schneider *et al*., 2011). Since also the grass intake (dry matter: 16.2 kg d^{-1}, SD 1.7 kg d^{-1}) was similar between cows, the δ^{13}C of the whole diet was very similar between cows (δ^{13}C -27.40 ‰, SD 0.45 ‰). The equilibration period was designed as a cross-over experiment to examine whether the results obtained in the stall can also be applied for grazing (Fig. 2). During half of the period, four cows were kept in the stall,

where grass intake could be measured (mass and isotopic composition), while the other four were kept on pasture except for the milkings, during which the cows also received the maize supplement. After half of the equilibration period, the groups were switched so that the cows kept on pasture were moved to the stall and *vice versa*.

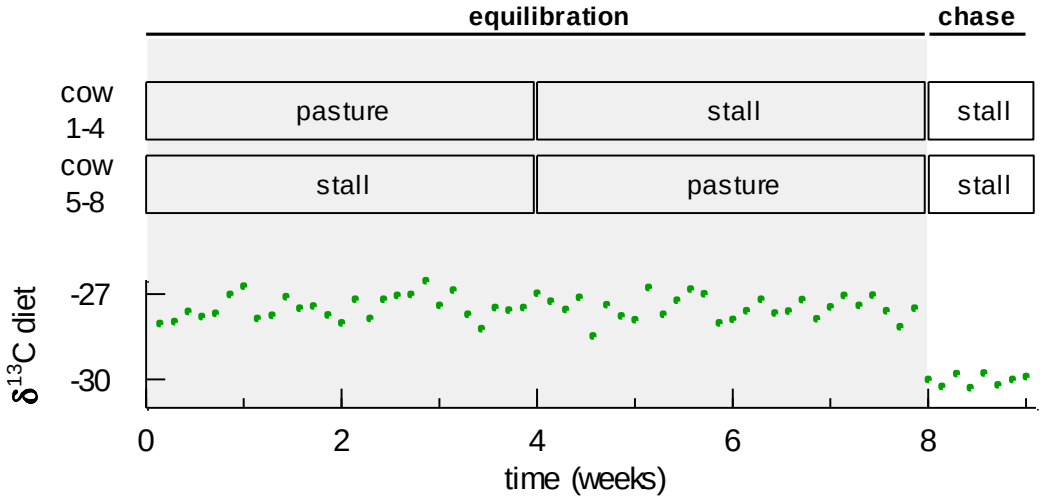

Figure 2: The experiment comprised an isotopic equilibration period lasting eight weeks and a chase period lasting ten days with different dietary $\delta^{13}C$. The equilibration period was conducted as a cross-over experiment during which two groups of cows were switched between stall and pasture.

An all-day pasture (3.0 ha, semi-natural grassland) with continuous stocking at 2.8 cow ha^{-1} was chosen, because it restricted selection during grazing and assured similarity of the grass fed on pasture and in stall. The target sward height for the all-day pasture was 6–7 cm and controlled daily (ca 150 measurements with a rising-plate meter from Ashgrove, RD 10, New Zealand) to prevent an ontogentic change in grass quality. To assure a similar diet for both groups of animals, grass for the stall group was harvested with a fodder harvester (Hege 212B, Waldenburg, Germany) between the grazing cows (fresh matter harvest approximately 450 kg d^{-1}). The grass was cut to a stubble height of 3 cm by assuming the 'take half' rule for mean bite depth (Woodward, 1998; Ungar & Ravid, 1999). Cutting was also used to maintain the target sward height when growing conditions allowed for more growth than consumed by the cattle. For more details see Schneider *et al.* (2011).

After eight weeks of equilibration, the maize supplement was eliminated from the diet, thus switching to a pure grass diet for the following ten days ('chase period').The equilibration period was six times longer than the chase period. This long equilibration procedure aimed at an isotopic equilibration of all body-pools that might contribute to the biosynthesis of milk components.

Sampling

Fresh grass and maize meal was sampled twice a day for each cow during the whole experiment. Grass samples were taken from the pasture grass that was harvested daily for feeding the cows in the stall. Further, milk, milk components and feces were sampled and analyzed for each cow four times a week (Monday evening, Tuesday morning, Thursday evening and Friday morning) during the equilibration period. The sampling interval was shortened during the chase period to quantify the isotopic turnover with greater accuracy (Fig. 3). Here, samples were collected twice daily (at each milking).

Feces and diet samples were oven dried (60 °C, 48 h) and milled. A subsample of whole milk was homogenized and freeze-dried. Another subsample was separated into milk fat, casein and lactose. First, milk fat was collected as the supernatant following centrifugation for 12 min at 2500 g. The casein was then precipitated by acidification to pH 4.3 with 10% HCl and subsequent centrifugation (30 min at 2500 g). The residue (whey), containing lactose and whey proteins, was heated to 80°C and filtered to remove the whey proteins and deliver lactose.

Statistical analysis

A delay between diet (input) switch and first output reaction was considered and compartmental mixed effects modelling was performed with the diet switch data. Compartmental modelling disentangles kinetically different pools contributing to an output (Martinez del Rio *et al*., 2009). Mixed effects models allow a statistically rigorous investigation of intra-population variability (Pinheiro *et al*., 2012). This approach comprised the following three steps:

First, samples belonging to the delay period were separated from samples belonging to the chase period. Samples were allocated to either period by stepwise testing sample t_0 (time at diet switch) against sample t_e (time e hours after diet switch) by applying the Wilcoxon matched pairs test. The first sample at t_e that was significantly different from sample t_0 was considered to be part of the chase period.

Second, the time course of the isotopic composition of the output ($\delta^{13}C_{output}$) during the chase period was analyzed by a compartmental mixed effects model. For compartmental modelling, multi-exponential decay functions

$$\delta^{13}C_{output} = c + \sum_{p=1}^{4} a_p \cdot \exp(-t/\tau_p) \qquad \text{(Eqn 2),}$$

were fitted to the data. The asymptotic base-line, which corresponds to the $\delta^{13}C$ resulting from the pure pasture grass diet, is represented by *c*. The index number of exponential terms (pools) is *p*. Parameter *a* depends on the initial deviation from *c*, and the relative contribution of the individual pools when the delay is subtracted from time *t*. The parameter τ_p is the mean residence time from which the half-life $t_{½}$ of pool *p* can be derived as $\tau_p \cdot \ln(2)$. The corresponding 95% confidence intervals were calculated as $t_{½} \pm SE \cdot T_a$, where SE is the standard error of estimated $t_{½}$ and T_a is the 97.5 percentile of the Student's t-distribution with n-1 degrees of freedom. The equation parameters are directly valid for independent pools. However, several layouts of interacting pools can be fitted with this equation, but the interpretation of the parameters is then not straightforward anymore (Martinez del Rio & Anderson-Sprecher, 2008). A pool may appear slow despite its fast turnover if it is not directly connected to the input and output. A decision on which layout of pools likely is valid cannot be made on statistical criteria due to the statistically similar performance of different layouts. It must be based on the physiological properties of the system (Schnyder *et al*., 2012). It will be shown that independent outputs are the most likely interpretation in this case.

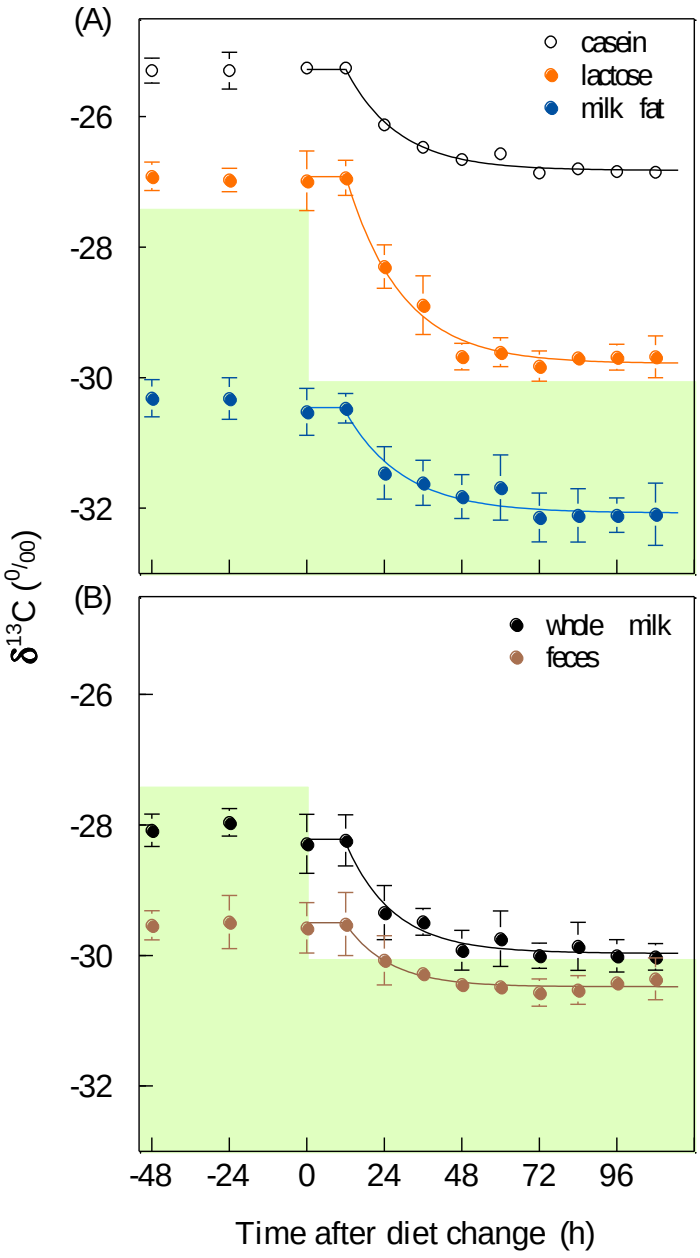

Figure 3: Time course of $\delta^{13}C$ in milk components (panel A) and in whole milk and feces (panel B) for a switch from a mixed diet to a pure grass diet. The mean diet $\delta^{13}C$ is shown in green. Error bars denote the 95% confidence intervals of the mean (n = 8 for each data point). Solid lines denote the turnover models, including a delay.

Models with up to four exponential terms were calculated. The number of exponential terms can be considered as the number of metabolic pools with different kinetic characteristics. Pools that differ biochemically but follow kinetics that cannot be resolved statistically are assigned to one pool, despite their biochemically contrasting nature. The mixed effects model technique accounted for fixed (time) and random (cows) factors in the compartmental models (for a detailed explanation of fixed and random factors see (Eisenhart, 1947). In particular, it allowed to decide on statistical criteria, whether the parameters a, τ_p and c were identical among all cows, or whether individual parameter estimates were advantageous. This permitted the simultaneous analysis of all eight cows, but avoided pseudoreplication, since repeated measures were obtained from the same individuals. Akaike's Information Criterion (AIC, Kuha, 2004) was then used to select the model best supported by the data among the models with different numbers of exponential terms and models with different random and fixed factors.

Third, the delay period was estimated by calculating the time at the intersection of the accepted model with the mean of $\delta^{13}C$ values of the previously estimated delay period. For comparison with other studies that ignored delays, a gross half-life was calculated. This represents the time after the diet switch (including the delay) at which half of the total output shift can be expected. In contrast to half-lives of individual pools, it applies only for the first half-life period but not later on because the delay would not be effective during the following half-life periods as long as there is no further diet switch.

To validate the milk component separation and measurement, milk $\delta^{13}C$ was back calculated from its components casein, lactose and milk fat for every milk sample accounting for the relative contribution of each component to the total carbon content of milk. The deviation between back calculated and original whole milk as estimated by the root mean squared error (RMSE) was negligible (0.09‰ for the mean values of the cows; the average SD was 0.4 ‰). This indicated that artefacts during separation and isotope analysis were likely small. Hence, original and back calculated whole milk had similar pool kinetics that were statistically not different (Table 5).

The applicability of the diet-switch based turnover models and their parameters to fluctuating diet isotope composition was tested with data from the equilibration period.

This took advantage of the naturally occurring short-term fluctuations in the $\delta^{13}C$ of the grass component during the equilibration period. Each output value at time "now" ($\delta^{13}C_{ouput;now}$) was calculated from previous input values starting six half-lives (referring to the maximum half-life of a multi-pool system) and the delay period before "now" according to

$$\delta^{13}C_{output;now} = \sum_{t=now-delay-6t_{½,max}}^{now-delay} (\delta^{13}C_{input;t} \cdot w_t) + \delta^{13}C_{shift} \qquad \text{(Eqn 3)},$$

where $\delta^{13}C_{input;t}$ is the input (dietary) isotopic signature at time t, and w_t is the weighting factor at time t within the considered time period from time "now – delay – $6t_{½,max}$" until "now – delay". Inputs of times greater than six half-lives of the slowest pool ($t_{½,max}$) were neglected, because they contribute < 0.01 %. The parameter $\delta^{13}C_{shift}$ indicates the trophic shift between input and output.

The weighting factor w_t results from Eqn 2 as

$$w_t = \frac{\sum_{p=1}^{P} a_p \cdot \exp(-t/\tau_p)}{\sum_{t=now-delay-6t_{½,max}}^{now-delay} \sum_{p=1}^{P} a_p \cdot \exp(-t/\tau_p)} \qquad \text{(Eqn 4)},$$

where the numerator is the value predicted by the compartmental model (Eqn 2, the constant offset parameter c can be neglected) for time t, and the denominator is the sum of values of the compartmental model during six half-lives for which inputs were considered. The parameter P is the number of previously estimated pools. For the measured output, the 95% prediction intervals were calculated as $\delta^{13}C_{output} \pm SD \cdot T_a$ (where T_a is again the 97.5 percentile of the Student's t-distribution) to examine whether the model output falls within the predicted range.

For the cows grazing on the pasture the same calculations were applied, except that the unknown grass intake was replaced by the average grass intake of the respective cow as measured during its stall period, while the same isotopic composition was assumed as that

measured for the same day for grass fed in stall. Hence, only maize intake and its isotopic composition were known. The predicted $\delta^{13}C$ of the outputs of the equilibration period for both stall and pasture cows were then compared with the independently measured $\delta^{13}C$ values.

The shift ε in $\delta^{13}C$ of outputs following the diet switch was calculated as

$$\varepsilon = (\delta_a - \delta_b)/(1+\delta_b)\cdot 1000 \quad \text{(Eqn 5)},$$

where the $\delta^{13}C$ before the switch (δ_b) was given by the $\delta^{13}C$ of the delay period, and the $\delta^{13}C$ after the switch (δ_a) was given by the fitted parameter c of Eqn 2, since c represents $\delta^{13}C$ after complete turnover.

Experiment 2: Quantifying isotopic turnover by inverse modelling of natural random fluctuations

Data acquisition

Isotopic carbon and nitrogen compositions were used from two published sources (Braun *et al*., 2013b, Schnyder *et al*., 2006). In total, 488 measurements of the diet and each 256 measurements of five products, namely whole milk, milk fat, casein, lactose and feces were available from the initial 6-wk long equilibration period of a diet-switch experiment that used lactating cows (Braun *et al*., 2013b). The isotopic carbon turnover of all products was known from curve fitting of the isotopic reaction progress in products following a systematic isotopic switch in the diet. Schnyder *et al*. (2006) used cows grazing on a pasture and provided each 24 measurements of diet and tail hair from one animal. The nitrogen turnover of the same animal had previously been determined by Schwertl *et al*. (2003) from the isotopic change induced by the switch from indoor to outdoor feeding after the start of the grazing season. However, the experiments by Braun *et al*. and by Schnyder *et al*. exhibited an extended period of rather constant diet without systematic switch, but with natural isotopic random fluctuations for carbon and nitrogen. This allowed the application of inverse modelling. The results of both methods could hence

be directly compared using data of the same products from the same animals within the same (Braun *et al*., 2013b) or a preceeding (Schwertl *et al*., 2003) experiment.

Additionally to the measurements, synthetic data were produced as they allowed changing the conditions of inverse modelling, like the source variance, the experimental error and the metabolism of hypothetic organisms (i.e. turnover parameters). Synthetic data were generated by repeating the following steps: (i) A source time series of normal distributed random data was generated (*S*, mean = 0, second order stationary, variance = *v*, number of data = *n*). The time scale of *S* is arbitrary, as it could be minutes, hours, days, weeks, *et cetera*, but the time scale of *S* determines the time scale of all procedures/outputs that are derived from *S* (e.g. the products calculated from *S* and the parameters delay and half-life of inverse modeling). Hence, *S* has "procedure defined units" (p.d.u., also called arbitrary units, see UIPAC), indicating that all procedures/outputs that are derived from *S* are *per definitionem* on the same (arbitrary) temporal scale as *S*, and pattern/results would be equal if all procedures/outputs were on a different scale. (ii) A product time series (*P*, number of data = *m*, source-product shift = 0) was calculated from *S* using a mono-exponential forward model (Eqn 7, see below). This allowed simulation of metabolic first order kinetic products for the given set of turnover parameters delay and half-life (Braun *et al*., 2013b). (iii) As *S* and *P* shall represent field data and such exhibit experimental errors (i.e. errors by sampling and measurement), experimental errors were simulated and added. To this end, normal distributed random data time series of the same length as *S* and/or *P* were added to *S* and/or *P* (mean = 0, variance = ε_s or ε_p). In case, experimental error was added to *S* and *P*, the error time series for *S* and *P* were independent. The signal-to-noise ratio (η) that quantifies the ratio of valuable variance (i.e. signal) to distorting variance (i.e. noise) was calculated as

$$\eta = \frac{v}{\sqrt{\varepsilon_S + \varepsilon_P}} \qquad \text{(Eqn 6)},$$

where *v* is the variance of isotopic source fluctuations that is obscured by the root of the sum of the variances of experimental errors in source and product (ε_s and ε_p), respectively (according to the Gaussian error propagation).

Data analysis

Inverse modeling was carried out by iteratively optimizing the parameters *d* (delay) and $t_{1/2}$ (half-life) of the equation

$$p_{sim;t+d} = \sum_{i=t-\omega t_{1/2}}^{t} (s_i \cdot w_i) + \Delta \quad \text{(Eqn 7)},$$

to minimize the root mean squared error (RMSE) between the simulated products (P_{sim}) and the measured products (P, see Fig. 4). The parameters s_i, Δ, and ω were predefined and denote the source data at times i, the source-product shift and a source integration factor, respectively. The source integration factor ω gives the number of half-lives over which s_i is integrated and determines the source integration time $\omega t_{1/2}$. The parameter w_j is the weighting factor for s_i that results from the first order kinetics. It is calculated as

$$w_i = e^{-i \cdot \ln(2)/t_{1/2}} \Big/ \sum_{i=t-\omega t_{1/2}}^{t} e^{-i \cdot \ln(2)/t_{1/2}} \quad \text{(Eqn 8)},$$

where the numerator is the value of the first order exponential reaction progress at time i, and the denominator is the sum of values of the first order exponential reaction progress during the source integration time. Notably, the forward model considers the effects of delay (*d)* and half-life ($t_{½}$), but neither by isotopic routing nor by growth. The source-product shift can be approximated as

$$\Delta = \frac{\sum_{i=1}^{m} p_i}{m} - \frac{\sum_{i=1}^{n} s_i}{n} \quad \text{(Eqn 9)},$$

where m is the number of product data, n is the number of source data. Eqn 9 should only be used when S and P have sufficient length to neglect the bias by the temporal shifts of P against S by delay and turnover (otherwise Δ has to be approximated from literature). The confidence intervals for d and $t_{½}$ are then given at the minimum RMSE + $SE \cdot T_a$, where SE is the standard error of the estimate and T_a is the 97.5 percentile of the Student's t-distribution with n-1 degrees of freedom.

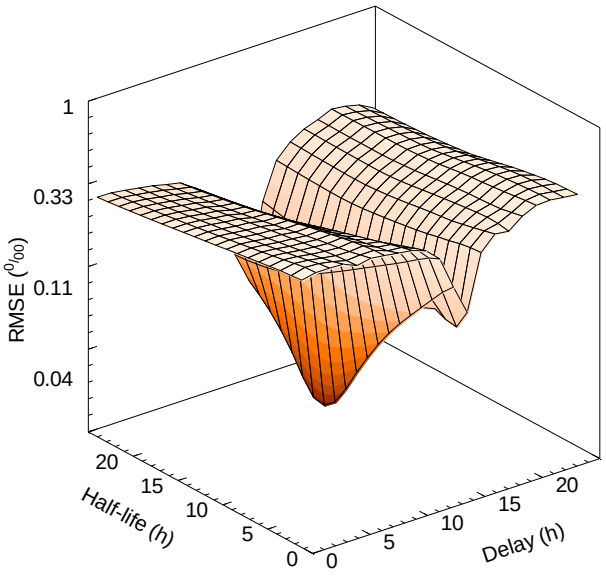

Figure 4: Illustrative example of inverse modeling for carbon turnover of milk: the root mean squared error (RMSE) is given by the combination of delay and half-life. The lowest RMSE indicates the most adequate combination of delay and half-life, here delay = 11 h and half-life = 18 h. Note the logarithmic z-axis.

An interpolation between measurements of S allows smaller time steps and may hence increases the precision of inverse modelling estimation (for parameter d and $t_{½}$). For the milk data, the measured source isotopic composition at a certain time was filled forward in steps of 1 h until the time of the next measurement, because the feeding protocol ensured that the source was constant until the next source was provided. In contrast, during the grazing experiment, the source (i.e. grassland diet) was expected to follow a trend in $\delta^{13}C$ between the samplings, e.g. due to variation in water use efficiency. Hence, data were interpolated in steps of 1 h under consideration of the temporal autocorrelation induced by such a trend (Piccini *et al*., 2014).

Evaluation of inverse modeling using synthetic data

Corresponding *S* and *P* were generated for $8.25 \cdot 10^5$ times, resulting from variation in delay (*d*, from 0 to 10 at interval 1), half-life ($t_{½}$, from 1 to 150 at interval 1) and signal-to-noise ratio (η, 0.5, 1, 10, 50 and 100), where each combination was repeated for 100 times. The parameter Δ was zero. For each repetition, the *d* and $t_{½}$ of *P* were estimated by inverse modelling and the deviation of the input to the inverse modeling output (denoted as

estimation error ε_x, either delay estimation error, ε_d, or half-life estimation error, $\varepsilon_{t½}$) was calculated as

$$\varepsilon_x = \sqrt{(X_{input} - X_{inverse\ modeling})^2} \quad \text{(Eqn 10)},$$

where X is either d or $t_{½}$ of the input or of the inverse modeling output. Non-linear regressions between mean ε_x (calculated from 100 repetitions) and potential drivers d, $t_{½}$ and η were calculated. In case, two horizontal asymptotes were apparent ($\lim_{x\to 0} b$ and $\lim_{x\to \infty} a$), the modified Gauss function

$$\varepsilon_x = (a-b)\left(1 - e^{\frac{-x^2}{c^2\frac{1}{3}}}\right) + b \quad \text{(Eqn 11)},$$

was fitted, where parameter a is the maximum for ε_x, the parameter b is the minimum for ε_x and parameter c is the value of x at which a is reached. In case, ε_x increased exponentially without reaching a horizontal asymptote within the considered range of x, the power function

$$\varepsilon_x = ax^b \quad \text{(Eqn 12)},$$

was fitted, where a is the intercept at x=1 and b is the positive rate constant. Tests on significant differences were done with Wilkinson matched-pairs test.

Experiment 3: Dietary protein content affects isotopic turnover

Animals and diet

The diet-switch experiment was performed with 18 rats (age of ~3 weeks and 50 g ± 5 g live weight at the beginning of the experiment). The rats were assigned to cages and maintained at 25°C ambient temperature, 60% relative humidity and a 12 h day – 12 h night cycle for 13 weeks. All rats had *ad libitum* access to deionized water supplemented with 0.014% NaCl and received a ration of 8 g day^{-1} (dry mass), consisting of 2.2 g sucrose, 0.6 g coconut fat, 0.3 g minerals, 0.2 g cellulose, 0.1 g sunflower oil, 0.02 g

vitamins and 0.02 g L-Methionine (Table 2). The remaining diet composition varied during the diet-switch experiment, as follows:

In order to equilibrate all rats to constant and equal isotopic signatures, the experiment started with a seven-week 'isotopic equilibration period'. The diet was supplied with 1.3 g (day 1-17), 1.0 g (day 18-34) and 0.8 g (day 35-49) casein, according to the protein requirements for juvenile rats. A ^{15}N labelled amino acid mixture similar in composition to the amino acid profile of casein was mixed at a ratio 1:100 with casein. However, aspartic acid and serine were substituted isonitrogenously by glycine, cysteine was substituted by methionine, and tyrosine was substituted by phenylalanine. L-proline was not available as an isotopically enriched amino acid. The diet was supplemented with 3.2 g, 3.5 g and 3.7 g starch, mixed of 25% corn starch and of 75% potato starch. The $\delta^{13}C$ value of the bulk diet did not exhibit a trend with changing diet compositions, because casein and starch (in this specific corn – potato ratio) had the same $\delta^{13}C$ value (Table 3). The $\delta^{15}N$ value of the bulk diet did not exhibit a trend with changing diet compositions, because the relation between ^{15}N amino acid mixture and casein was constant.

Table 2: Diet compositions during the experiment

	Isotopic equilibration period			**Chase period**	
	day 1-17	day 18-34	day 35-49	day 50 - 92	
				Low protein	High protein
	g kg^{-1} dry mass				
Casein	160	130	100	100	300
^{15}N amino acids	1.6	1.3	1	0	0
Starch (corn:potato)	406 (1:3)	436 (1:3)	467 (1:3)	468 (1:0)	268 (1:0)
Sucrose	280	280	280	280	280
Fat	70	70	70	70	70
Minerals	37.5	37.5	37.5	37.5	37.5
Cellulose	30	30	30	30	30
Oil	10	10	10	10	10
Vitamins	2.5	2.5	2.5	2.5	2.5
DL-Methionine	2	2	2	2	2

At day 50, the six-week 'chase period' started and each batch of nine animals was randomly assigned to one of two experimental groups. The protein content of the 'low protein' group remained at 10%, whereas the protein content of the 'high protein' group increased to 30% (supplementation of starch was accordingly decreased to 26%). In both groups, the starch composition changed to 100% corn starch; thus the bulk dietary $\delta^{13}C$ value changed from -22.9 ‰ to -18.4 ‰ ± 0.2 ‰ in the 'low protein' group, and changed to -20.3 ‰ ± 0.2 ‰ in the 'high protein' group. The diet of the 'high protein' group was slightly depleted in ^{13}C compared to the diet of the 'low protein' group, because casein was more depleted in ^{13}C than starch from maize, and the casein/starch ratio was higher. The supplementation of the ^{15}N amino acid mixture stopped and the bulk dietary $\delta^{15}N$ value changed from 1761 ‰ to 5.5 ‰ ± 0.1 ‰, independent of the experimental group (Table 3).

Table 3: Isotopic compositions during the experiment. Measurements are shown ± standard deviation. n. d. = not determined.

	$\delta^{13}C$ (‰)	$\delta^{15}N$ (‰)
Diet components:		
Casein	-23.1 ± 0.1	4.6 ± 0.1
Amino acid mixture	n.d.	177500 ± n.d.
Starch mixture	-23.1 ± 0.2	
Bulk diets:		
Equilibration diet	-22.9 ± 0.1	1761 ± 21.0
"Low protein" diet	-18.4 ± 0.2	5.5 ± 0.1
"High protein" diet	-20.3 ± 0.2	5.5 ± 0.1

Sampling

One week prior to the chase period the rats were transferred to single-rat metabolic cages that allowed the separate collection of urine and feces. Excreta were collected individually for each animal. Microbial adulteration of the urine was prevented by providing 2 mL of 6 N H_2SO_4 in the urine collectors that were renewed daily.

The diet and the excreta were collected daily, starting 5 days before the 'chase period' to day 7 of the chase period and additionally on days 10, 13, 17, 22, 27, 34, 41 of the chase period. At the start of the 'chase period', four rats (two of each group) were sacrificed under anaesthesia to obtain isotopic reference values that were not influenced by the diet-switch. During the 'chase period' one rat of each experimental group was sampled on days 2, 7, 13, 20, 27, 34, 41 to investigate the reaction progress of the isotopic composition following the diet switch. The animals as a whole and their organs were weighed to the nearest g. Their blood was collected into heparinized tubes, transferred to glass tubes and centrifuged for 20 min at 1207 x *g*' (4°C) (MSE, Lorch/Wittenberg, Germany) to obtain blood plasma. The animals were dissected to provide the organs muscle, liver, heart, spleen, kidney, lung and brain. All organs were then stored at -80°C. Before analysis, organs and excreta were thawed and homogenized by an Ultra-Turrax (IKA-Labortechnik Jahnke & Kunkel GmbH & Co KG, Staufen i. Br., Germany). For the isotope analysis of muscle tissue, musculus femoris and musculus quadriceps were combined, homogenized with an Ultra-Turrax, rinsed with a solution of hexane-isopropyl alcohol (3:2) and filtrated. The eluted muscle fat was separated from the dispersant using a rotary evaporator (Buchi, Flawil, Switzerland). The remaining fat-free muscle and the other organs and excreta were dried for 12 h at 70°C.

Calculations and Statistics

The dietary nitrogen uptake was calculated as the daily nitrogen mass in the diet on offer minus the nitrogen mass in the diet refused by the individual animals. The nitrogen net balance is then given as nitrogen uptake minus fecal and renal nitrogen excretion.

The isotopic turnover of each organ or excrement was analyzed separately for each isotope and each experimental group. A delay between time of diet-switch and first order reaction progress that is likely due to transit time was considered. Neglecting the delay would cause overestimation of half-lives (Cerling *et al*., 2007). To this end, a continuous but non-differentiable, piecewise linear/nonlinear function was fitted to the data, where the linear function at the beginning represents the delay and the following nonlinear function represents the reaction progress, from which the isotopic turnover rate is derived. The

reaction progress is determined by the isotopic renewal rate of organs or excreta, which is the isotopic turnover rate *sensu stricto*, and optionally by organ growth (Fry & Arnold, 1982). Thus, it was first evaluated whether or not organs were growing by regressing the organ's fresh masses against time. In case of no growth, the reaction progress directly indicates the turnover rate and the function

$$\delta X_{(t)} = \begin{cases} \delta_{initial} & for\, t \in [0; d[\\ \delta_{\infty} + (\delta_{initial} - \delta_{\infty}) e^{-r(t-d)} & for\, t \in [d; \infty[\end{cases} \qquad \text{(Eqn 13),}$$

was fitted to the data, where $\delta X_{(t)}$ is the isotopic signature (either $\delta^{13}C$ value or $\delta^{15}N$ value) at time t, $\delta_{initial}$ is the initial isotopic signature that is constant during the delay period, δ_{∞} is the asymptotic isotopic signature after complete turnover, r is the turnover rate constant and d is the delay. In case of growth, the reaction progress does not directly indicate the turnover rate, but the effects of growth and turnover can be disentangled by fitting a 'mass model' as suggested by Carleton & Martinez del Rio (2010). Their 'mass model` was extended with the metabolic concept of a delay (according to Eqn 13), leading to the function

$$\delta X_{(t)} = \begin{cases} \delta_{initial} & for\, \Delta w_t / w_t \in [0; \Delta w_d / w_d[\\ \delta_{\infty} - (\delta_{\infty} - \delta_{initial})(1 - \Delta w_t / w_t + \Delta w_d / w_d)^{(1+r/g)} & for\, \Delta w_t / w_t \in [\Delta w_d / w_d; \infty[\end{cases}$$

(Eqn 14),

where w_t and w_d are the organ`s mass at time t and time d, respectively, Δw_t is w_t - w_0, Δw_d is w_d - w_0 and g is the exponential growth rate constant, estimated as $g = \ln(w_t/w_0)/t$ (Ebert, 1999). The ratio r/g gives the relative contribution of isotopic turnover (r) and growth (g) to the apparent reaction progress. The ratio $\Delta w_d/w_d$ can be transformed into the delay. Since $t = \ln(w_t/w_0)/g$, and $w_d = -w_0/((\Delta w_d/w_d)-1)$, the delay is given by substitution of w_t by w_d, which simplifies to $\ln(-1/(\Delta w_d/w_d-1))/g$ and can reasonably be approximated by w_d/g for small w_d. For both models, the parameter $\delta_{initial}$ was obtained by measuring the initial isotopic signatures (as described above), as this value characterizes the constant isotopic signature during the delay and simultaneously the intercept of the following reaction

progress model when delay time is subtracted. The delay (d) was iteratively changed from zero to ten days in steps of 0.1 days, and in case of growth, $\Delta w_d/w_d$ was changed from zero to 0.2 in steps of 0.002 leading to 100 models in both cases. For each of these, the remaining two parameters (δ_∞ and r) were fitted. The half-life ($t_{½}$) could be calculated from r as,

$$t_{½} = \ln(2)/r \qquad \text{(Eqn 15).}$$

The root mean squared error (RMSE) between model ($\delta X_{(t)}$, either Eqn 13 or Eqn 14) and measured data was calculated for each of the 100 models differing in d and $\Delta w_d/w_d$, respectively, and thus in δ_∞ and r, as

$$RMSE_{\delta X} = \sqrt{\sum_{t=0}^{k}(\delta X_{(t)} - \delta m_{(t)})^2 / n} \qquad \text{(Eqn 16),}$$

where $\delta m_{(t)}$ are the measured data at times t, $\delta X_{(t)}$ are the modeled data at times t obtained either from Eqn 13 or from Eqn 14, k is the maximum sampling duration and n is the number of samples. The model with the lowest RMSE was accepted. Tests on significant differences of d and $t_{½}$ between ‘low protein’ and ‘high protein’ group and between elements were done with Wilkinson matched-pairs test. A test on significant differences of d between all organs and excreta was done with Kruskal-Wallis test. One sample t-test was done to test the mean of all d on significant difference from zero.

Experiment 4: Transamination reduces heterogeneity in isotopic nitrogen composition of amino acids in animals

Animals and ^{15}N labelling

This experiment used the same animals from the same diet-switch experiment as "Experiment 3: Dietary protein content affects isotopic turnover". However, to understand the isotopic labelling, the following additional information is important: An amino acid mixture that was similar to the amino acid profile of casein, but highly enriched in ^{15}N (atom fraction 0.95 or 0.99) was added to the diet, approximately at a ratio 1:100 to casein (Table 4). However, aspartic acid and serine were substituted isonitrogenously by glycine, cysteine was substituted by methionine, and tyrosine was substituted by phenylalanine. Also proline was not added as an isotopically enriched amino acid. This led to a large variation in labelling of the individual amino acids in the diet with the atom fractions of ^{15}N ranging from 0.0037 to 0.0332 (Table 4). This pronounced labelling was chosen because any fractionation by the metabolism will be much smaller and can thus be neglected. For example, biosynthesis of glutamic acid from aspartic acid causes fractionation (Macko *et al*., 1987), but this will lower the atomic fraction only by ~ 0.0036 and thus does not interfere with the labelling, which caused an atom fraction in the labelled diet of 0.0220.

Calculation of $x(^{15}N)$ of dietary amino acids

The $x(^{15}N)$ of the amino acids in the bulk diet was calculated according to mass balance of an amino acid provided by casein and provided by the enriched amino acids of the labelling mixture, as

$$x(^{15}N) = (m_{cas} \cdot \mathrm{x}(^{15}N)_{cas} + m_{label} \cdot \mathrm{x}(^{15}N)_{label})/(m_{cas} + m_{label}) \qquad \text{(Eqn 17)},$$

where m_{cas} and m_{label} are the masses of the specific amino acid provided by casein and by the labeled amino acid mixture, respectively, while $x(^{15}N)_{cas}$ and $x(^{15}N)_{label}$ are the corresponding $x(^{15}N)$.

Table 4: Amino acid profile in dietary casein and in the amino acid labelling mixture and the resulting atom fraction of ^{15}N ($x(^{15}N)$) in the bulk diet. The $x(^{15}N)$ of amino acids in the bulk diet is calculated from mass balance and a $x(^{15}N)$ of 0.004 for casein and 0.950 for the enriched amino acids except for Tyr and Try, for which $x(^{15}N)$ was 0.990.

Amino acids	Content of amino acids in bulk diet from		$x(^{15}N)$ of amino acids in
	Casein	Label mixture	Bulk diet
	($g\ g^{-1}$)		
Alanine	5	0.02	0.008
Arginine	2.5	0.01	0.007
Asparagine	4.1	0	0.004
Aspartic acid	2.2	0	0.004
Cysteine	0.8	0	0.004
Glutamine	7.7	0	0.004
Glutamic acid	9.3	0.18	0.022
Glycine	2.2	0.07	0.033
Histidine	1.9	0.03	0.016
Isoleucine	5.7	0.05	0.012
Leucine	9.1	0.08	0.012
Lysine	7.2	0.04	0.009
Methionine	4.6	0.05	0.013
Phenylalanine	3.9	0.05	0.016
Proline	9.8	0	0.004
Serine	7.4	0	0.004
Threonine	5.7	0.05	0.011
Tryptophan	0.7	0.01	0.017
Tyrosine	4.2	0	0.004
Valine	7.3	0.06	0.011

Sampling

Sampling was essentially identical to "Experiment 3: Dietary protein content affects isotopic turnover". However, since it was intended to investigate individual amino acids, rather than bulk tissues, the following steps had to be taken additionally:

Samples of 2.5 – 3.5 mg muscle lyophilisate were double-washed with 10% trichloroacetic acid, acetone and ether. Washed samples were hydrolysed (24 h, 2 ml 6 N HCl at 110°C), derivitized (N-Pivaloyl-i-Propyl-Ester), dried and solved in 100 µl pyridine. Then, 100 µl of pivalochloride were added, the solution was acetylated (30 min, 60 °C), cooled and 2 ml CH_2Cl_2 were added. The filtrate was dried under a stream of N_2 and 100 µl ethylacetate was added. The samples were then analysed on a gas-chromatograph (HP 5890, Waldbronn, Deutschland), connected to a gas-isotope-ratio-mass spectrometer (Finnigan MAT, Bremen, Deutschland), via a GC-combustion-interface.

Turnover estimation

The previous study that used the same animals had shown that the reaction progress of all organs including the muscle exhibited a common delay of 0.5 d following a diet-switch, which likely was caused by the transit time in the digestive tract (Braun *et al*., 2013a). Since neglecting a delay would lead to an overestimation of half-lives, the delay (0.5 d) was subtracted from the sampling times to provide a delay-corrected reaction progress.

Since growth affects the reaction progress (Carleton & Martinez del Rio, 2010) and the muscle from which the amino acids were sampled had a growth rate of ~ 0.7 g d^{-1} (Braun *et al*., 2013a), the effects of growth and turnover rate were disentangled from the delay-corrected reaction progress by fitting the function

$$x(^{15}N)_{(t)} = x(^{15}N)_{\infty} - (x(^{15}N)_{\infty} - x(^{15}N)_0)(1 - \Delta w / w_t)^{(1+r/g)} \quad \text{(Eqn 18)},$$

where $x(^{15}N)_t$ is the atom fraction at time t, $x(^{15}N)_{\infty}$ is the atom fraction of the asymptote at the end of the reaction progress and $x(^{15}N)_0$ is the atom fraction at time zero (time of isotopic switch plus delay). The parameter w_t is the dry mass of an amino acid at time t and

Δw is w_t - w_0, where the subscript 0 denotes time zero. The exponential growth rate constant is denoted g and estimated for each amino acid specifically as $0.7/\gamma$ g d^{-1}, where γ was the relative abundance of an amino acid in the muscle. The ratio r/g gives the relative contribution of isotopic turnover (r) and growth (g) to the apparent reaction progress. The half-life ($t_{½}$) is then given by $\ln(2)/r$. The parameter $x(^{15}N)_0$ was obtained by measuring the isotopic composition at the end of the 'equilibration period' (as described above), as this value characterizes the constant isotopic composition during the delay and simultaneously the intercept of the following reaction progress model when delay time is subtracted (Fig. 5).

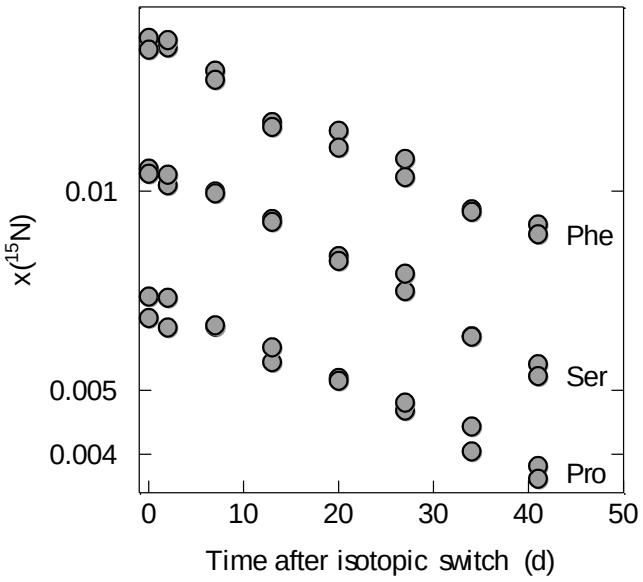

Figure 5: The reaction progress of the atom fraction $x(^{15}N)$ of amino acids phenylalanine (Phe), serine (Ser) and proline (Pro). Each of these belongs to one of three hypothesized groups. Since the y-axis is logarithmic, the similar slopes indicate similar turnover rates.

Experiment 1:
Forward modeling of fluctuating dietary ^{13}C signals to validate ^{13}C turnover models of milk and milk components from a diet-switch experiment

A similar version of this chapter was published as:

Braun A, Schneider S, Auerswald K, Bellof G, Schnyder H (2013) Forward Modeling of Fluctuating Dietary ^{13}C Signals to Validate ^{13}C Turnover Models of Milk and Milk Components from a Diet-Switch Experiment. PLoS ONE 8(12): e85235. doi:10.1371/journal.pone.0085235

Alexander Braun analyzed the data, discussed the results, composed the graphs and tables, developed the story of the paper, wrote the first draft of the paper and revised the paper.

Abstract

Isotopic variation of food stuffs propagates through trophic systems. But, this variation is dampened in each trophic step, due to buffering effects of metabolic and storage pools. Thus, understanding of isotopic variation in trophic systems requires knowledge of isotopic turnover. In animals, turnover is usually quantified in diet-switch experiments in controlled conditions. Such experiments usually involve changes in diet chemical composition, which may affect turnover. Furthermore, it is uncertain if diet-switch based turnover models are applicable under conditions with randomly fluctuating dietary input signals. Here, it was investigated if turnover information derived from diet-switch experiments with dairy cows allows predicting the isotopic composition of metabolic products (milk, milk components and feces) under natural fluctuations of dietary isotope and chemical composition. First, a diet-switch from a C_3-grass/maize diet to a pure C_3-grass diet was used to quantify carbon turnover in whole milk, lactose, casein, milk fat and feces. Data were analyzed with a compartmental mixed effects model, which allowed for multiple pools and intra-population variability, and included a delay between feed ingestion and first tracer appearance in outputs. The delay for milk components and whole

milk was ~12 h, and that of feces ~20 h. The half-life ($t_{½}$) for carbon in the feces was 9 h, while lactose, casein and milk fat had a $t_{½}$ of 10, 18 and 19 h. The ^{13}C kinetics of whole milk revealed two pools, a fast pool with a $t_{½}$ of 10 h (likely representing lactose), and a slower pool with a $t_{½}$ of 21 h (likely including casein and milk fat). The diet-switch based turnover information provided a precise prediction (RMSE ~0.2 ‰) of the natural ^{13}C fluctuations in outputs during a 30 days-long period when cows ingested a pure C3 grass with naturally fluctuating isotope composition.

Introduction

Isotopic variation in food stuffs propagates through trophic systems generating isotopic tags in organisms at each trophic level (DeNiro & Epstein, 1978). Metabolically active tissues steadily degrade and renew and thus integrate dietary isotopic signals (Ayliffe *et al*., 2004). Propagation of natural dietary isotopic variation was found in wildlife and livestock species, and also in humans (Bowen *et al*., 2005; Schwertl *et al*., 2003; Wright, 2012). For carbon, such dietary variation may result from differences in carbon isotopic discrimination between plants with different photosynthetic mechanisms and variation within each mechanism resulting from species-specific morpho-physiological properties and responses to environmental drivers (Farquhar *et al*., 1989; Hao *et al*., 2011). Moreover, post-photosynthetic fractionation phenomena can generate variation in carbon isotope composition ($\delta^{13}C$) of chemical compounds and plant parts (Tcherkez *et al*., 2011).

A delay can be expected from feed intake to first appearance of dietary isotopic signals in tissues (Cerling *et al*., 2007), due to ingestion and passage time. The rate of incorporation of "new" diet isotopes and simultaneous loss of "old" isotopes in tissues of consumers is then driven by metabolic processes, such as cycling and storage, and can be comprehensively quantified as isotopic turnover. Turnover implies that isotopic variation in consumer tissues is attenuated, meaning that the isotopic composition of the tissue fluctuates less than that of the diet (Passey & Cerling, 2002). The degree of attenuation is inversely related to turnover rate; thus, attenuation is weak if turnover is fast and strong if turnover is slow.

Turnover of tissues in different animal species, here assessed as half-lives, range from < 1 d (Pearson *et al*., 2003) to > 100 d (Bahar *et al*., 2009; Martinez del Rio & Carleton, 2012). Half-lives are modified by endogenous and exogenous factors: in particular, half-lives differ between species (e.g. cattle and rats, Bahar *et al*., 2009; Kurle, 2009), organs/tissues (e.g. liver and blood, Miller *et al*., 2008) and between chemical compounds (e.g. carbohydrates and proteins, Martineau *et al*., 1985). For example, the carbon isotopic half-lives vary between muscle tissue of cattle and rats by 120 d, because isotopic turnover rate declines with body mass following the -¼ power (Martinez del Rio *et al*., 2009). Half-lives also vary within the same species between different tissues, e.g. liver and muscle of *Rattus norvegicus* by 30 d (Kurle, 2009), likely as a result of differences in protein turnover (Martinez del Rio *et al*., 2009). Moreover, half-lives vary with growth (Fry & Arnold, 1982), temperature (Witting *et al*., 2004), diet composition (Haramis *et al*., 2001; Pearson *et al*., 2003; Miron *et al*., 2006), sex and age (Lecomte *et al*., 2011). In particular, the carbon isotopic half-life of blood in nectarivorous bats varied by 60 % following a change in the diet (Miron *et al*., 2006).

Half-lives are assessed in isotopic or diet switch experiments (Boutton *et al*., 1988; Hobson & Clark, 1992; Bahar *et al*., 2009). Such experiments involve a systematic shift of the isotopic composition of the diet, recording of the time course of the isotopic composition in the outputs of interest and compartmental modelling of the tracer data. Compartmental modelling has frequently been used to investigate the kinetic properties of metabolic and storage pools (Martinez del Rio *et al*., 2009; Lattanzi *et al*., 2012), because it allows inferring the number of kinetically different pools in a metabolic system, and their specific pool half-lives and relative sizes. In diet switch experiments, animal and environmental conditions as well as diets are usually strictly controlled in order to realize close-to-steady-state conditions, except for the isotopic composition of the diet that is altered at the time of the switch. In principle, the steady-state conditions are violated if the isotopic switch involves a switch in diet chemical composition, as is frequently the case (this study, Boutton *et al*., 1988; Knobbe *et al*., 2006; Miron *et al*., 2006; Sponheimer *et al*., 2006; Alves-Stanley & Worthy, 2009; Bahar *et al*., 2009). Such non-steady state conditions, in combination with fluctuating environmental conditions, question the validity of diet-switch based turnover parameters and data. It is a difficult – if not impossible – task

to maintain the chemical composition of the diet while changing its isotopic composition, particularly in studies with large herbivores, as is also the case in the present study. For instance, replacement of C_3 grasses by C_4 grasses, including maize, generates a strong shift in $\delta^{13}C$, but it also means a shift from fructan-dominated to fructan-free non-structural carbohydrate contents (Chatterton *et al*., 1989) and a shift in feed tissue structure and anatomy (e.g. Krantz anatomy, van Soest, 1994; Wilson, 1994). Switches between grasses and legumes usually involve changes in protein content, tissue composition, and cell wall structure and chemistry (van Soest, 1994). Yet, essentially all of our knowledge on isotope turnover in large mammals, particularly herbivores, has been derived from diet-switch experiments of the above type. For this reason, there is an interest in the validation of diet-switch based turnover information under conditions differing from estimation. In particular, it is of interest to know if such models – derived under near-constant conditions – can predict the propagation of natural fluctuations in diet isotope composition to the products/outputs like milk. Fluctuations in the composition of feed are likely to be associated with changes in digestibility. Feces should hence show opposite behaviour to metabolic products, as a large proportion of the feces does not pass the gastrointestinal barrier to become part of the inner body, particularly the systemic circulation, but moves through the digestive tract as undigested residue (Wingfield & Raffe, 2002). Comparing metabolic products with feces thus increases the sensitivity for validating turnover models.

In this study with dairy cows, it was investigated if diet-switch based turnover information (i.e. models and their parameters) allows the predicting of isotopic composition of metabolic outputs (milk, milk components and feces) at different dietary conditions that showed naturally fluctuating $\delta^{13}C$. To this end, the changes of $\delta^{13}C$ in whole milk, lactose, casein, milk fat and feces were assessed in a diet-switch experiment. The data were analyzed with a compartmental mixed effects model, which allowed for multiple pools and intra-population variability. The model also included a delay between ingestion and first appearance of tracer in whole milk, milk components and feces. Then, the model of the whole milk was validated by back calculating whole milk from its components, fitting a model to the back calculated whole milk and comparing this model with the model of the original milk. The back calculation was done by adding the measured values of all three components according to their relative contribution to the carbon content

of the whole milk. Finally, the performance of the models in predicting the $\delta^{13}C$ of outputs at different dietary conditions was assessed.

Results

Isotopic characterization of inputs and outputs

The $\delta^{13}C$ of the diet (fresh pasture grass and maize meal) and of whole milk, milk components (lactose, casein, milk fat) and feces collected from eight cows over nine weeks (including the equilibration and the chase period) provided a total of 2013 samples and isotope analyses. This included 133 samples of inputs (diet) and 1880 samples of outputs (milk, milk components or feces). The output samples divide into 47 samples per output per cow, with 32 samples from the equilibration period and 15 samples from the chase period.

The $\delta^{13}C$ of inputs and outputs showed no significant trend over the equilibration period. The $\delta^{13}C$ of inputs also showed no significant trend over the chase period. Nevertheless, naturally occurring short-term fluctuations were apparent in the inputs at both periods (SD 0.45 ‰ in equilibration and 0.38 ‰ in chase period) and also in the outputs (0.15 ‰) over the equilibration period. During the equilibration period, $\delta^{13}C$ was -29.06 ‰ (SD 0.43 ‰) for grass and -12.14 ‰ (SD 0.21 ‰) for maize meal. Given the contribution of grass (90.2 %, SD 0.6 %) and maize meal (9.8 %, SD 0.6 %) to the diet, the $\delta^{13}C$ of the input was -27.40 ‰ (SD 0.45 ‰). The carbon and nitrogen contents of the input during the equilibration period were 45.4 % (SD 0.6 %) and 3.2 % (SD± 0.4 %) respectively. Milk fat (-30.23 ‰, SD 0.67 ‰), feces (-29.51 ‰, SD 0.52 ‰) and whole milk (-28.02 ‰, SD 0.48 ‰) were isotopically depleted in ^{13}C compared to the input, whereas lactose (-26.94 ‰, SD 0.38 ‰) and casein (-25.39 ‰, SD 0.67 ‰) were isotopically enriched in ^{13}C compared to the input. The $\delta^{13}C$ of the input changed to -30.04 ‰ (SD 0.38 ‰) with the start of the chase period, creating an input switch of 2.7 ‰. The carbon and nitrogen contents of the input during the chase period were 41.8 % (SD 0.6 %) and 4.0 % (SD 0.4 %), respectively.

Compartmental mixed effects modelling

The $\delta^{13}C$ of outputs did not change immediately after the input switch but exhibited a delay. Usually the first two measurements, one at the switch, the other at 12 h later, did not differ significantly from the mean $\delta^{13}C$ of the equilibration period. Thus, these samples were included in the delay period. Delay periods, calculated as the time of intersection between the turnover model with the mean $\delta^{13}C$ during the equilibration, were relatively short for casein, lactose, milk fat, whole milk and back calculated milk (about 12 h), and longer for feces (20 h, Table 5).

After the delay, the $\delta^{13}C$ of all outputs changed rapidly to reach a near-constant value after two to three days. The $\delta^{13}C$ of the last four measurements could no longer be distinguished, indicating that the changes within these two-day periods were smaller than the confidence interval based on the experimental error and the number of replicates. The shift in $\delta^{13}C$ following the diet switch (-2.7 ‰) differed among outputs. It was largest in lactose (-2.9 ‰, SD 0.1 ‰) and smaller in whole milk (-2.1 ‰, SD 0.1 ‰), milk fat (-2.0 ‰, SD 0.3 ‰), casein (-1.6 ‰, SD 0.1 ‰) and feces (-1.1 ‰, SD 0.1 ‰).

The AIC statistics supported only one pool for each milk component and for the feces (Table 5). Half-lives were short for feces and lactose (9 h and 10 h) and longer for casein and milk fat (18 h and 19 h). Conversely, whole milk was best fitted with a two-pool model including a fast pool (10 h) and a slow pool (21 h). The half-life of the fast pool was not statistically different from that of lactose (but differed from that of fat and casein), while the half-life of the slow pool was statistically indistinguishable from milk fat and casein (but differed from lactose). The gross half-life was short for lactose (22 h) and longer for feces, whole milk, casein and milk fat (approx. 30 h).

The individual cows followed the same chase kinetics with the same parameter values as indicated by the mixed effects models. Also, no difference was apparent between the cows that grazed and those that were in stall immediately before the diet switch.

Table 5: Compartmental model parameters for the outputs feces, milk components, whole milk and back calculated whole milk that was calculated from the milk components: pool number, delay, pool half-life and gross half-life resulting from the delay and the pool half-lives; 95 % confidence intervals of the mean are given in parenthesis.

Output	Pool	Delay (h)	Pool half-lives (h)	Gross half-lives (h)
Feces	1	20 (19 – 22)	9 (6 - 12)	29
Lactose	1	12 (10 – 13)	10 (8 - 12)	22
Casein	1	12 (10 – 14)	18 (15 - 21)	30
Milk fat	1	12 (10 – 14)	19 (17 - 21)	31
Whole milk	1	12 (10 – 15)	10 (8 - 12)	28
	2		21 (18 - 24)	
Back calculated whole milk	1	12 (10 – 14)	11 (8 - 14)	28
	2		22 (18 - 26)	

Predicting isotopic fluctuations of outputs

The dietary input $\delta^{13}C$ varied randomly with a range of 2 ‰ during the equilibration period (Fig. 2). This variation was short-term and some variation of $\delta^{13}C$ was also found in the outputs, but the variation there was attenuated and delayed. The output variation predicted from the input by forward modelling is shown, as an illustrative example, for milk fat and lactose of two different cows in Fig. 6A (n = 16 for each output and each animal). The modelled and the measured $\delta^{13}C$ of the outputs for all animals kept in stall arrange around the 1:1 line (Fig. 6B). None of the slopes differed statistically significantly from one, and none of the intercepts differed statistically from zero when the measured values of the outputs were separately regressed against their predicted values. The RMSEs were ~ 0.2 ‰ (RMSE for whole milk 0.29 ‰, for lactose 0.21 ‰, for milk fat 0.22 ‰, and for feces 0.24 ‰) and within the confidence intervals of the measurements, except for casein, which exhibited a larger unexplained variation (RMSE 0.43 ‰). For the

animals kept on pasture, this was essentially also true (RMSE increased by about 0.06 ‰ for all outputs, data not shown), when the unknown grass intake was replaced by the average grass intake of the respective cow as measured during its stall period and, the same $\delta^{13}C$ was assumed for the grass intake as that measured for the same day for grass fed in stall.

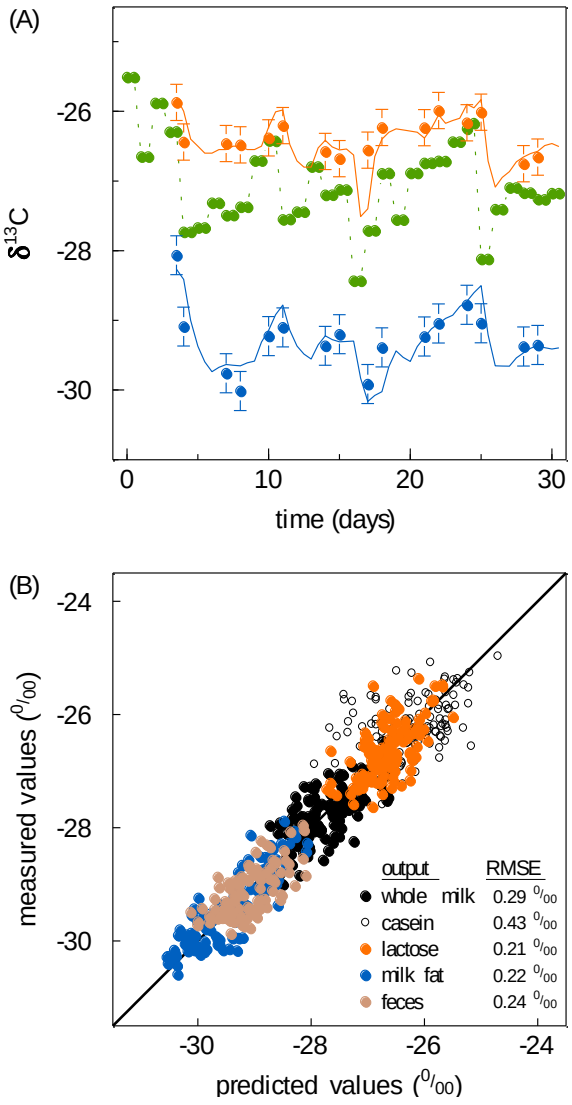

Figure 6: Panel A shows fluctuations of $\delta^{13}C$ in diet (green circles, dashed line) and of the products milk fat (blue circles) and lactose (orange circles) as obtained from measurements during the equilibration period and predictions by the compartmental model for milk fat and lactose (blue and orange line) obtained during the chase period. Error bars denote the 95% confidence intervals of the mean. Panel B compares all measured and predicted $\delta^{13}C$ values of whole milk and components (casein, lactose and milk fat) and feces; n = 128 for each output. The 1:1 line is represented by a solid line.

Discussion

Diet-switch based turnover models allowed the predicting of fluctuating isotopic compositions of metabolic outputs. This worked similarly well for outputs differing strongly in their genesis, namely milk, milk components and feces. Despite their differing genesis, all outputs were kinetically rather similar. Delays, comprising ingestion, rumen and intestine passage, were short, but – although principally known (e.g. Zazzo *et al*., 2008) – to our knowledge not previously quantified and integrated into turnover models for lactating ruminants. As pointed out by Cerling *et al*. (2007) neglecting the effects of these delays will cause erroneous turnover estimation, especially, when delay and half-life are similar in length. In our case, the gross half-life (delay plus metabolic half-life) thus overestimated the true metabolic half-life by about factor two (Table 5).

The turnover of all outputs was fast, especially compared to bovine muscle tissue ($t_{½}$ ~ 150 d, Bahar *et al*., 2009). Nevertheless, significant differences among outputs were evident. The order of half-lives of milk components (lactose < casein < milk fat) was consistent with two other studies (Kleiber *et al*., 1952; Klein *et al*. 1986), despite substantial differences between study designs (breed, diet, tracer injection site). The combination of short delays and short half-lives make milk and feces valuable non-destructive short-time indicators for food authentication and animal ecology that allow inferring the $\delta^{13}C$ of the diet from 12 to 50 hours prior to milking (i.e. delay plus two half-lives, Hobson & Clark, 1992).

Compartmental analysis identified two pools in milk that were not a combination of a fast and a slow pool supplying synthesis, but were due to the combination of three products differing in half-lives (with two being similar in half-life) as shown with the back calculated milk. An interpretation of multi-pool models is thus not straightforward in the case of heterogeneous products derived from different sources. Thus, this study also indicated that the statistical component separation cannot replace their analytical separation (Cheeseman, 1986).

Predicting isotopic fluctuations of outputs

The good performance of the turnover models as derived from the chase period for predicting the isotopic variation during the equilibration period (Fig. 6) implies that all differences between both periods had little influence, namely the temporal type of isotopic variation (fluctuation vs switch), source of isotopic variation (grass vs maize), and ambient conditions (stall vs pasture), which potentially affect turnover (e.g. Witting *et al*., 2004). This good performance may be unexpected, especially because the diet differed between chase and equilibration period. Therefore it was concluded that the change in diet and the associated change in nutritional value were small enough not to confound the isotopic turnover. Two reasons to support this conclusion will be discussed: (i) the animals were kept at nutritional maintenance and thus milk and feces synthesis were almost entirely derived from exogenous sources, and (ii) the ruminal host-microbe interaction caused isotopic scrambling:

(i) At a level of nutrition below maintenance there is depletion of body stores, which then become an additional, endogenous source that enters the pool turnover for output synthesis (Ayliffe *et al*., 2004). If this were to occur it would need to be considered in the prediction. However, animal weights did not change. Therefore milk synthesis – within the detection limit of the study – was derived from the diet and pool turnover. This agrees with Boutton et al. (1988) and Martin & Sauvant (2007) who found that milk synthesis in mid and end lactation (for cows fed at maintenance, as herein) was almost exclusively derived from exogenous sources. The same should be the case for feces.

(ii) Diet changes have been found to affect half-lives in non-ruminants (Haramis *et al*., 2001; Pearson *et al*., 2003; Miron *et al*., 2006). In contrast, the ruminal host-microbe interaction converts dietary material into microbial biomass that is utilized (Edwards *et al*., 2008). This biomass is quite stable to diet switches as long as diet switches are small enough not to affect the ruminal or post-ruminal turnover (Susmel *et al*., 1991; Russel & Rychlik, 2001), which is likely to have been the case given the near-identical digestibility of the two rations (Schneider *et al*., 2011).

The model validation was only possible because the equilibration period, which was intended to provide isotopically constant feed, showed a pronounced variation and that a record of the dietary input existed. It is beyond the scope of this study to identify the

reasons for the fluctuations in the grass feed. However, ample possibilities for such fluctuations exist, as it is known that isotopic composition differs among species even of one photosynthetic type (Hao *et al*., 2011) and hence among parts of a grassland differing in species composition (Wittmer *et al*., 2010), but also with short-term fluctuations in water availability (Wittmer *et al*., 2008) and with ontogenesis (Gessler *et al*., 2008; Hao *et al*., 2011), among others. Hence it is likely that in studies where no continuous record of the dietary input existed, such fluctuations also occurred and obscured the turnover parameters. Even in the case of homogenized and manufactured feed, strong fluctuations may appear (Zazzo *et al*., 2007). Therefore, continuous diet records are needed in isotope turnover studies or studies quantifying the isotopic shift (Auerswald *et al*., 2010). This may even offer new approaches to turnover quantification that avoid the artificial diet switch.

Different isotopic shifts between outputs

The isotopic shifts caused by the diet switch were consistently higher in the milk outputs than in feces. The shift was largest in lactose. This indicated isotopic routing (Schwarcz, 1991). The starch from maize meal is known to be partly ruminally protected and can pass the rumen without degradation and incorporation into microbial biomass (Nocek & Tamminga, 1991). The ruminally protected starch is then enzymatically broken down to glucose in the small intestine, absorbed and largely metabolized to lactose. Hence, lactose exhibited a slightly larger shift (-2.9 ‰) than the shift in diet (-2.7 ‰) while the opposite was the case for the casein (-1.6 ‰), which is produced from the microbial biomass. The even smaller shift in feces likely was caused by the slightly lower digestibility of the grass than the maize (Schneider *et al*., 2011) increasing the proportion of grass residues in the feces.

The discrepancies between the diet shift and the output shifts will also cause a variation of diet-to-output discrimination ("trophic shift"). This implies that these discriminations vary with diet type but even for the constant diet type during the equilibration period, the diet-to-output discrimination apparently varied due to the delay and attenuation of the isotopic signal as indicated by the fluctuating distance between the input and the outputs in Fig. 6A. Also discrimination between different outputs (e.g. between lactose and milk fat) will vary if delay and/or half-life vary between both outputs.

Mathematically this must cause correlations between discrimination and the isotopic composition of the diet, which have frequently been found, but which are spurious (Auerswald *et al*., 2010). The correlations do not indicate an influence of the isotopic composition itself, but indicate that the output is not in isotopic equilibrium with the input, which hinders the proper quantification of the input-output discrimination.

Conclusions

Diet switch-based turnover models allowed a successful prediction of fluctuating isotopic output signatures at times beyond parameter estimation. This indicated that the diet change by omitting a small C_4 component did not affect the turnover leading to milk, lactose, milk fat and feces. Milk was described best by a two-pool model, which was caused by the different half-lives of its components, while the contribution of different pools during synthesis could not be shown for any of the analysed milk components. The short half-lives caused the recent diet to dominate all products. A significant fluctuation during the intentionally constant equilibration period allowed the validation of the turnover model but indicated that continuous feed records are essential in studies quantifying turnover or trophic shifts.

Experiment 2:
Quantifying isotopic turnover by inverse modelling of natural random fluctuations

Abstract

Isotopic variation propagates from source to product. In particular, the isotopic signature of a product is the delayed integration of past dietary isotopic signatures. The delay between incorporation and integration is determined by transit and synthesis time and the integration is determined by the turnover time of the product, herein quantified as isotopic half-life. Hence, when the past isotopic signatures of the source and the turnover parameters (i.e. delay and half-life) are known, the isotopic signatures of the product can be predicted by forward modelling. The turnover parameters should be inferable by inverse modelling, using corresponding isotopic source and product data that exhibit fluctuations.

Using published data, inverse modelling was applied to isotopic random fluctuations of the diet and the results were compared to that of systematic isotopic switch experiments following exponential curve fitting. Using synthetic data, the influence of turnover parameters and signal-to-noise ratio on the estimation errors of delay and half-life of inverse modelling was evaluated.

The turnover information from well-planned diet-switch experiments and of inverse modelling were in mutual agreement. This offers the chance to use inverse modelling instead of diet-switch experiments, especially with wildlife species given that the natural fluctuation is sufficiently high (e.g. seasonal variation). The power of inverse modelling depends on turnover parameters and the signal to noise ratio. In particular, estimation errors decreased with increasing variance of fluctuations and decreasing half-life of the product.

Introduction

Dietary nutrients supply renewal and (incremental) growth of organismic materials (Ayliffe *et al*., 2004, Martinez del Rio *et al*., 2009). Hence, the isotopic composition of an organismic material is the delayed integration of dietary isotopic composition modified by fractionation and thus materials provide a log of past dietary input (DeNiro & Epstein, 1978; Braun *et al*., 2013b). The integration time is determined by the renewal (i.e. turnover) time of the material and its growth (Martinez del Rio *et al*., 2009). Additionally, a delay between incorporation and integration can be expected due to transit and synthesis time (Cerling *et al*., 2007).

Knowledge on delay and turnover (herein quantified as half-life), provides information on metabolic state and health of organisms (Fearon *et al*., 1988; Berclaz *et al*., 1996), as used in physiological and medical investigations; and allows the enlightening of trophic relationships (Auerswald *et al*., 2010; Codron *et al*., 2012, Layman *et al*., 2012), geographical origin and migration patterns (Trueman *et al*., 2012), as used in ecology, forensics and food authentication.

Isotopic turnover is usually assessed in isotopic switch experiments (e.g. Hobson & Clark, 1992; Miron *et al*., 2006; Bahar *et al*., 2009). Such experiments involve a systematic switch of source isotopic composition inducing an exponential reaction progress in the product isotopic composition (Fig. 7), from which the isotopic turnover is derived by fitting an exponential curve (hence denoted curve fitting). Controlled experimental conditions are required providing steady-state of factors that potentially affect the organisms' metabolism and thus the reaction progress, e.g. environmental conditions, like temperature (Witting *et al*., 2004) or the source chemical composition (Tsahar *et al*., 2007, Braun *et al*., 2013a). Even changes in source chemical composition beneath the threshold of metabolic effects are to be avoided, because they may cause (non-systematic) isotopic variation (i.e. fluctuations) in source that is propagated to the products (Braun *et al*., 2013b, Fig. 7) and potentially interferes the curve fitting as unexplained variance. Such isotopic fluctuations should occur in grassland diets that provide the nutritional base for many organisms: the isotopic signature differs among species even of one photosynthetic type (Tcherkez *et al*., 2011) and even within one grassland organism due to short-term

fluctuations in water availability (Wittmer *et al*., 2008) and ontogenesis (Gessler *et al*., 2008; Hao *et al*., 2011), among others.

However, the metabolic processes responsible for the propagation of source fluctuations have been mathematically characterized by an exponential forward model (Braun *et al*., 2013b); fluctuations are attenuated and temporally shifted, where the degree of attenuation increases with half-life and the delay determines the temporal shift. Thus, if the source fluctuations and the isotopic turnover are known, the fluctuations of the product can be simulated. In case, isotopic turnover is not known, but the fluctuations of source and product are, the isotopic turnover should be assessable by inverse modeling. Inverse modeling calibrates the forward model by an appropriate optimization algorithm that minimizes an objective function, i.e. searching for the best set of parameters (delay and half-life) of the forward model in an iterative way, by varying the parameters and comparing the real response of the system measured during an experiment (here the product) with the numerical solution given by the forward model (Ritter *et al*. 2003). Inverse modeling could hence open isotopic turnover assessment to close-to-natural conditions, as it does not imply systematic isotopic switch experiments tantamount with strict dietary control and artificial animal keeping. Inverse modeling could be particularly important for organisms that suffer from artificial keeping, especially wildlife animals, but would be also applicable to 'controlled environments' where isotopic fluctuations occurred.

Herein, the feasibility of inverse modelling is demonstrated by application to published data sets that contain products like milk and hair of which the isotopic turnover is known. Further, the influence of turnover parameters and signal-to-noise ratio on the estimation errors of delay and half-life of inverse modelling was evaluated, using synthetic data.

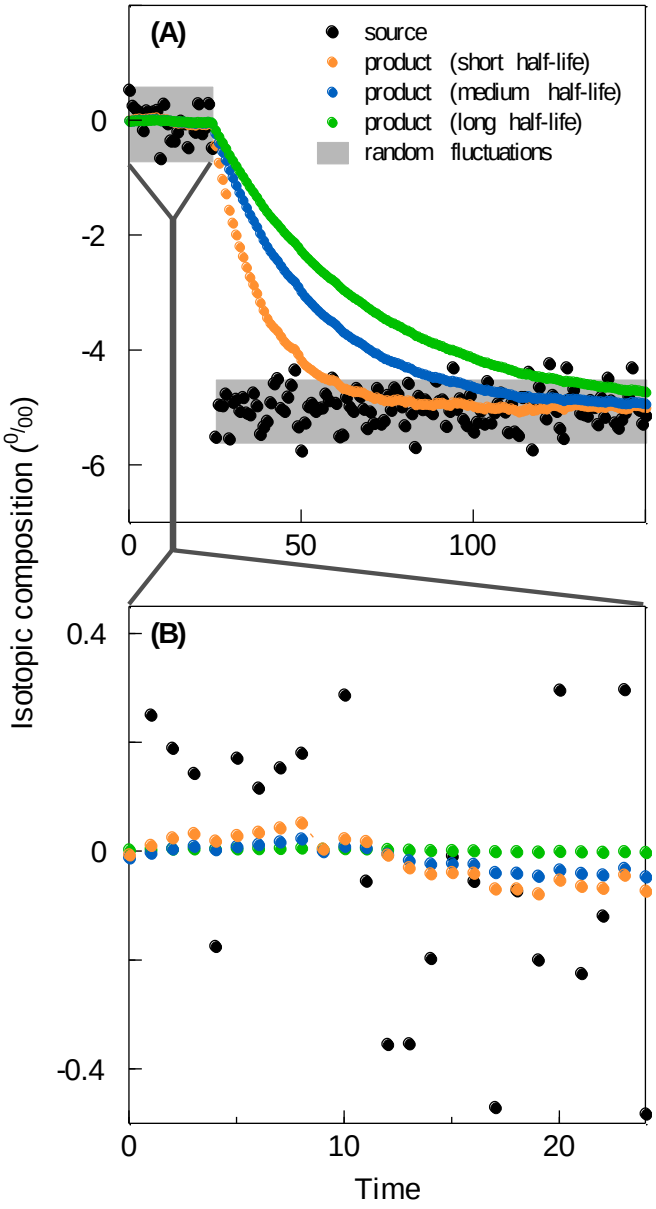

Figure 7: The isotopic reaction progress of products caused by a systematic switch (Panel A) and random fluctuations (Panel B) in isotopic composition of the source. Products differ in half-lives. Panel B exaggerates the period of random fluctuations before the switch. All data were simulated; the source-product shift was set to 0 ‰.

Results

Applying inverse modeling to the data of Braun et al. (2013b)

The variance in the diet was 0.43 ‰ for $\delta^{13}C$ and 0.52 ‰ for $\delta^{15}N$. The variances in the products were attenuated; they were 0.27 ‰ for $\delta^{13}C$ and 0.32 ‰ for $\delta^{15}N$ in feces, 0.24 ‰ for $\delta^{13}C$ and 0.11 ‰ for $\delta^{15}N$ in milk, 0.15 ‰ for $\delta^{13}C$ in lactose, 0.25 ‰ for $\delta^{13}C$ and 0.18 ‰ for $\delta^{15}N$ in casein and 0.35 ‰ for $\delta^{13}C$ in milk fat. The variance was induced by (random) fluctuations, because neither the isotopic signatures of the diet, nor of the products showed a significant trend. Casein and lactose were isotopically enriched in ^{13}C compared to the diet by 1.1 ‰ (0.7 ‰ – 1.6 ‰) and 0.7 ‰ (0.3 ‰ – 1.1 ‰), respectively, feces and milk fat were isotopically depleted in ^{13}C by 1.7 ‰ (1.2 ‰ – 2.2 ‰) and 2.2 ‰ (1.6 ‰ – 2.8 ‰), respectively, whereas whole milk had no significant shift. Milk and casein were isotopically enriched in ^{15}N compared to the diet by ~ 3.1 ‰ (2.6 ‰ - 3.6 ‰), and feces was enriched in ^{15}N by 2.3 ‰ (1.9 ‰ - 2.7 ‰). The isotopic turnover derived by inverse modeling revealed a significant and consistent delay in both isotopes that was ~12 h for milk (components), but ~22 h in feces (Table 6). The half-lives ranged from 9 h to 20 h in all materials. Neither the delays nor the half-lives were significantly different between inverse modeling and curve fitting, when considering their confidence intervals.

Applying inverse modeling to the data of Schnyder et al. (2006)

The variance in diet was 0.31 ‰ for $\delta^{13}C$ and 0.72 ‰ for $\delta^{15}N$. The variance in hair was attenuated; it was 0.21 ‰ for $\delta^{13}C$ and 0.43 ‰ for $\delta^{15}N$. Hair was isotopically enriched in ^{13}C compared to the diet by 0.8 ‰ (0.6 ‰ - 1.0 ‰) and enriched in ^{15}N by 2.2 ‰ (2.0 ‰ - 2.4 ‰). A negative trend was apparent in diet and hair for both isotopes, however isotopic fluctuations were apparent. The isotopic turnover derived by inverse modelling showed a common delay of ~ 2 d and half-lives of 12 d and 14 d for carbon and nitrogen turnover, respectively. The nitrogen half-life obtained by inverse modeling was significantly shorter than the nitrogen half-life obtained by curve fitting, when considering their confidence intervals.

Table 6: Delays and half-lives of carbon and nitrogen isotopic turnover of different materials estimated by inverse modeling and by curve fitting. Confidence intervals are in parentheses. The results quantified by curve fitting are from Braun *et al*. 2013b and Schwertl et al. 2003, respectively.

Material	Isotope	Inverse modeling		Curve fitting	
		Delay	Half-life	Delay	Half-life
Milk	C	11 h (8 h - 13 h)	18 h (11 h - 20 h)	13 h	10 h & 24 h
	N	12 h (10 h - 19 h)	9 h (2 h - 17 h)		
Lactose	C	12 h (7 h - 18 h)	14 h (5 h - 18 h)	12 h	11 h
Milk fat	C	16 h (11 h - 19 h)	19 h (12 h - 26 h)	18 h	20 h
Casein	C	15 h (8 h - 24 h)	20 h (12 h - 26 h)	12 h	19 h
	N	12 h (9 h - 16 h)	9 h (1 h - 16 h)		
Feces	C	21 h (13 h - 25 h)	10 h (5 h - 15 h)	20 h	9 h
	N	23 h (16 h - 29 h)	11 h (8 h - 14 h)		
Hair	C	2 d (1 d - 4 d)	12 d (10 h - 15 h)		
	N	2 d (1 d - 5 d)	14 d (11 h - 17 h)		19 d

Evaluation of inverse modelling using synthetic data

When synthetic data were free of experimental error (i.e. ε_s and $\varepsilon_p = 0$, for a list of abbreviations, see Table 7), the estimates of inverse modelling for the parameters d and $t_{½}$ were free of error (i.e. ε_d and $\varepsilon_{t½} = 0$). When experimental error was added to the data (i.e. ε_s and $\varepsilon_p > 0$), the estimates for d and $t_{½}$ deviated from their input values (ε_d and $\varepsilon_{t½} > 0$), where ε_d and $\varepsilon_{t½}$ were inversely related to the signal to noise ratio (Fig. 8). The ε_d ranged from ~0.01 p.d.u. to 10 p.d.u. and the $\varepsilon_{t½}$ ranged from ~0.01 p.d.u. to 150 p.d.u., comprising the full possible range with regard to the constraints of evaluation (see methods). The ε_d and $\varepsilon_{t½}$ were higher by 65% when experimental error was added to product data (*P*) but not to source data (*S*), than if the same error was added to *S* but not to *P*. The ε_d and $\varepsilon_{t½}$ were additionally higher by 10 %, when experimental error was added to

S and *P*. Since having experimental errors in *S* and *P* is a likely scenario in practice, the further evaluation considered equal experimental errors in *S* and *P*. It turned out, that ε_d and $\varepsilon_{t½}$ were influenced by signal to noise ratio (η) and half-life ($t_{½}$), whereas the delay (d) had no effect.

Table 7: List of abbreviations.

Abbreviation	Meaning
S	source data
P	product data
d	delay
$t_{½}$	half-life
ε_s	experimental error in the source data
ε_p	experimental error in the product data
ε_d	estimation error in delay estimation
$\varepsilon_{t½}$	estimation error in half-life estimation
η	signal to noise ratio
p.d.u.	process define units

Influence of η and $t_{½}$ on ε_d and $\varepsilon_{t½}$: The ε_d remained rather constant at shorter $t_{½}$, then increased exponentially with increasing $t_{½}$, before turning into a constant maximum (Fig. 8). The $\varepsilon_{t½}$ increased exponentially with increasing $t_{½}$, without turning into a constant maximum for longer $t_{½}$ (within the considered range of $t_{½}$,). Although both pattern were generally valid (represented by Eqn 11 and Eqn 12, respectively), a higher η lead to a lower ε_d and ε_t, respectively.

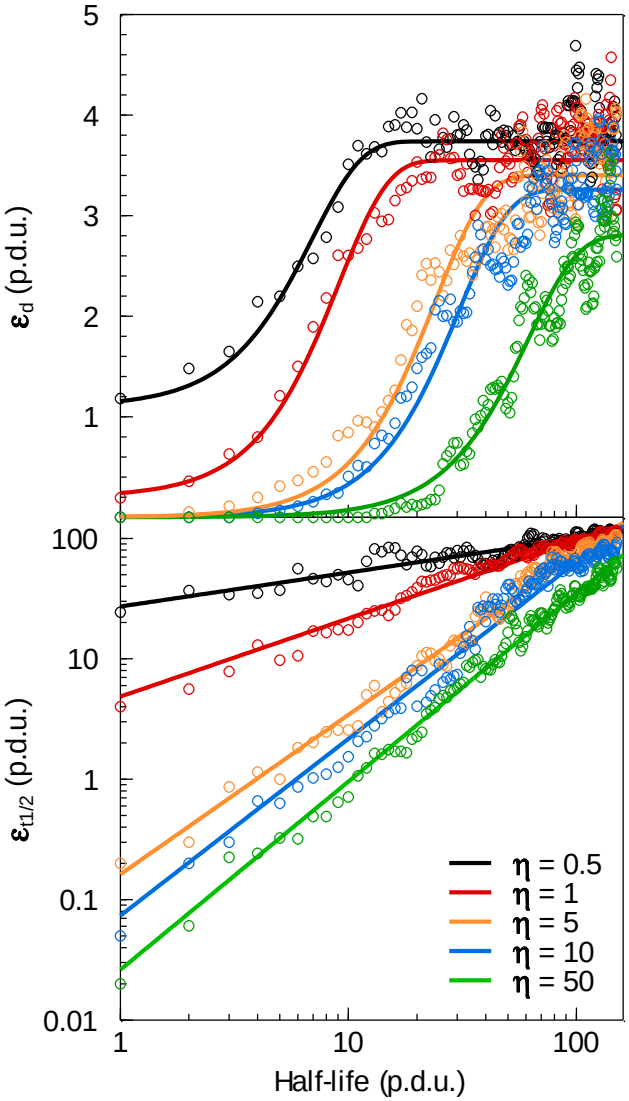

Figure 8: Estimation errors of the delay (ε_d) and of the half-life ($\varepsilon_{t½}$) depending on the half-life of the product and on the signal to noise ratio (η), when η was influenced by equal error in source and product. The open points are the means of ε_d and $\varepsilon_{t½}$ from the repeated simulations (n = 100). Lines denote the non-linear regressions. P.d.u. denotes process defined units, as the same pattern appears independent of the temporal scale of half-life, ε_d and $\varepsilon_{t½}$, as long as all three parameters share a common temporal scale, e.g. minutes, hours, days etc.

Discussion

Inverse modeling allowed quantifying isotopic turnover, i.e. delay and half-life, when the isotopic compositions of source and product fluctuated. Since isotopic fluctuations should occur frequently in natural or close-to-natural feeding regimes, e.g. in grassland diet (Schulze *et al*., 2006; Wittmer *et al*., 2010; Tcherkez *et al*., 2011), inverse modelling should be considered an alternative to systematic isotopic switch experiments (Hobson & Clark, 1992; Bahar *et al*., 2009). In particular, the possibility of omitting systematic changes in source isotopic composition that usually imply changes in dietary compositions should be an advantage, because such changes could impact animal behavior, treatment and physiology (Miron *et al*., 2006; Tsahar *et al*., 2007; Braun *et al*., 2013a) and thus potentially interfere turnover quantification. Inverse modelling could also be applied to wildlife animals that can hardly be kept in artificial environments as it potentially allows quantifying isotopic turnover without interacting with the animal, given that diet (e.g. grassland) and product (e.g. feces or hair) can be sampled at sufficient accuracy and assigned to one animal (e.g. by DNA analysis, Hopwood *et al*., 1996). The simultaneous quantification of delay and half-life avoids biased half-life estimates that could occur when a delay is neglected (Cerling *et al*., 2007).

The evaluation of inverse modelling using synthetic data showed that this method was most suitable for products with rather short half-lives (with respect to the time scale, Fig. 8). The estimation errors for delay and half-life were two orders of magnitude smaller for products with half-life 10 p.d.u. (~0.2 for delay and ~1 for half-life at $\eta = 10$) compared to products with half-life 100 p.d.u. (~3 and ~100, respectively). The (random) fluctuations of the source were more conserved in products with short half-life, because their source integration time was shorter and hence the contributions of individual sources had a higher impact. In contrast, products with longer half-life had more attenuated, thus more similar patterns, which tended to represent the mean signature of the source (± trophic shift) without variation. A precise differentiation between several "high half-life patterns" was thus more afflicted with estimation errors.

No significant differences in delays and half-lives were apparent between the results of inverse modelling and curve fitting (Table 6), when curve fitting accounted for a

delay (compare Braun *et al*., 2013b). In contrast, when curve fitting did not account for a delay (compare Schwertl *et al*., 2003), the half-life of inverse modeling was significantly shorter than in curve fitting, because neglecting a delay leads to overestimation of half-lives (Cerling *et al*., 2007). When the data of Schwertl *et al*. (2003) were reanalyzed and a delay was included in curve fitting, the difference in half-life was no longer significant (results not shown). Hence, both estimation methods provided similar results, though differing in the type of isotopic variation used (random fluctuations vs systematic switch).

The potential of inverse modeling to apply to or even to disentangle multi-compartment systems remains rather unknown. However, inverse modelling – which considers only pool kinetics, so far – estimated a single half-life of ~18h for milk, which was previously found to follow two-pool kinetics (Braun *et al*., 2013b). Interestingly, the single half-life of inverse modelling was the mean of the individual half-lives of the two-pool model from compartmental modelling.

Experimental errors in products affected the estimation errors of inverse modelling more by 65% than experimental errors in the source. Source data are integrated and thus non-systematic random errors (as used in the evaluation) should compensate (to a certain amount), whereas product data are not integrated. Hence, the product should be sampled and measured very carefully, e.g. doing triplicates, which can also be used to quantify the measurement error and to calculate the signal-to-noise ratio.

Experiment 3:

Dietary protein content affects isotopic carbon and nitrogen turnover

A similar version of this chapter was published as:

Braun, A., Auerswald, K., Vikari, A. and Schnyder, H. (2013), Dietary protein content affects isotopic carbon and nitrogen turnover. Rapid Commun. Mass Spectrom., 27: 2676–2684. doi: 10.1002/rcm.6737

Alexander Braun analyzed the data, discussed the results, composed the graphs and tables, developed the story of the paper, wrote the first draft of the paper and revised the paper.

Abstract

RATIONALE - Isotopic turnover quantifies the metabolic renewal process of elements in organs and excreta. Knowledge of the isotopic turnover of animal organs and excreta is necessary for diet reconstruction via stable isotope analysis, as used in animal ecology, palaeontology and food authentication. Effects of dietary protein content on the isotopic carbon and nitrogen turnover (i.e. delay, representing the time between ingestion and start of renewal, and half-life) are unknown for most mammalian organs and excreta.

METHODS - To examine the effect of dietary protein content on turnover (delay and turnover rate), 18 rats were fed either a diet at protein maintenance or above protein maintenance, their isotopic carbon and nitrogen turnover in ten organs and excreta was quantified. These included the excreta feces and urine, the visceral organs blood plasma, liver, kidney, lung and spleen, the cerebral tissue brain, and the muscular tissues heart and muscle. For data analysis, piecewise linear/non-linear exponential modelling that allows quantifying delay and turnover rate simultaneously was used.

RESULTS - Delays were ~0.5 days for carbon and nitrogen turnover and were not affected by dietary protein content. Half-lives during the following reaction progress were in the range of 1 to 45 days, increasing from excreta to visceral organs to muscular and cerebral

organs. Rats fed the higher protein amount had 30% shorter nitrogen half-lives, and 20% shorter carbon half-lives.

CONCLUSIONS - The renewal times of organs and excreta depend on the dietary protein content. Hence, isotopic diet reconstruction is confronted with variation in half-lives within the same organ or excrement, altering the time window through which information can be perceived.

Introduction

Most organismic materials steadily degrade and renew (Ayliffe *et al*., 2004). Such materials are organs, like liver and muscle, and excreta, principally feces and urine. The renewal is supplied by "old" nutrients from body-internal recycling but also by "new" dietary nutrients – hence, "we are what we eat, isotopically" (DeNiro & Epstein, 1978). Systematically switching the isotopic composition of the diet induces an exponential isotopic reaction progress in organs and excreta that can be quantified as isotopic turnover (Sponheimer *et al*., 2006; Lecomte *et al*., 2011). A delay between isotopic switch and reaction progress can be expected due to mastication, digestive transit, passing the gastro-intestinal barrier, transportation in the systemic circulation and delayed synthesis of new components from precursors (Cerling *et al*., 2007). The reaction progress is determined by the renewal rate, but can be obscured by growth (Fry & Arnold, 1982); growth will apparently shorten the reaction progress, because of a net addition of new material (with the new signature) simultaneously to renewal. However, adequate statistical analysis that considers growth rate allows disentangling the apparent reaction progress into fractional contributions of growth and of renewal and provides the true renewal rate (Carleton & Martinez del Rio, 2010), denoted as half-life.

The half-life can differ among elements within the same organ or excrement (Miron *et al*., 2006), e.g. among carbon and nitrogen isotopes, because different elements are subject to different renewal processes. Carbon turnover is affected by carbohydrate, fat and protein turnover, whereas nitrogen turnover is mainly connected to protein turnover

(MacAvoy *et al*., 2005). In particular, the nitrogen turnover integrates the processes of (i) formation and transamination of amino acids, (ii) biological protein synthesis, i.e. arrangement of dietary amino acids during translation according to transcripted mRNA in the ribosomes, (iii) incorporation of these proteins into a specific organ and (vi) degradation following pathways such as the cytosolic ubiquitin pathway to proteosomes, the degradation of defective proteins in the endoplasmic reticulum and lysosomal degradation (Johnson *et al*., 1999).

Knowledge on the isotopic turnover (i.e. delay and turnover rate) provides researchers with the time window through which they can perceive information when analysing the isotopic signature of an organ or excrement (Martinez del Rio *et al*., 2009). This is frequently used for diet reconstruction, providing time- and space-integrated insights into trophic relationships (Layman *et al*., 2012), natural diet switches (Cerling *et al*., 1999), geographical origin and migration patterns (Trueman *et al*., 2012) and food authentication (Bahar *et al*., 2008), covering the fields of animal ecology, palaeontology and food safety. Also, the quantification of diet-tissue shifts requires consideration of the turnover (Auerswald *et al*., 2010; Codron *et al*., 2012, Woodland *et al*., 2012).

Delays have been found in birds and sheep (Cerling *et al*., 2007; Zazzo *et al*., 2008), ranging from three days to two weeks. Drivers of this variation are not explicitly yet known, but any process/structural property that affects the transit from ingestion to incorporation should affect the delay. The fibre content of the diet has been found to affect the digestive transit time (Raczynski *et al*., 1982), indicating the potential of the delay to vary with diet composition. Conceptual or statistical limitations may prevent the quantification of a delay, e.g. the most frequently applied non-linear exponential modelling can not, per se, account for a delay (Martinez del Rio & Anderson-Sprecher, 2008). This can lead to inadequate turnover information, in particular to overestimation of half-life, and obscure the diet reconstruction (Cerling *et al*., 2007; Zazzo *et al*. 2008).

Isotopic half-lives in different animal species range from 1 d, e.g. in birds (Pearson *et al*., 2003), to > 100 d, e.g. in cattle (Bahar *et al*., 2009). Within one organism, the half-lives vary between organs, e.g. liver and muscle (Miller *et al*., 2008) and between chemical

compounds, e.g. carbohydrates and proteins (Martineau *et al*., 1985). Within each of these units, the half-life can vary with temperature (Witting *et al*., 2004), sex and age (Lecomte *et al*., 2011) and diet (Haramis *et al*., 2001; Pearson *et al*., 2003; Chamberlain *et al*., 2006). From physiological studies it is known that dietary protein content has a regulatory effect on protein synthesis and degradation (Lobley 2003; Waterlow, 2006), e.g. increased protein intake significantly increases the rate of whole body protein turnover (Pannemans *et al*., 1995; Bowtell *et al*., 1998; Gaine *et al*., 2006) and hence the renal hemodynamics, including glomerular filtration rates (Friedman, 2004). On the level of organs and excreta, an effect of dietary protein content on isotopic nitrogen turnover was found in blood of bats and birds (Voigt *et al*., 2003; Miron *et al*., 2006; Tsahar *et al*., 2007). However, there are no published studies on the effects of dietary protein content on isotopic turnover in other mammalian organs and excreta and on other isotopes. It was hypothesized that such an effect is not restricted to blood, because all cellular proteins are in a dynamic state of renewal (Waterlow, 1984); and not restricted to nitrogen isotopes, because carbon supports the backbone of proteins (Berg *et al*. 2010). However, the effect may differ between carbon and nitrogen isotopes, because carbon and nitrogen turnover integrate different renewal processes. In contrast, growth was found to be unaffected by varying protein intake that is beyond malnutrition (Tarnopolsky *et al*., 1988) and so should be the nitrogen net balance, because the renal excretion rate adapts to protein intake.

The effect of dietary protein content on the isotopic carbon and nitrogen turnover (i.e. delay and turnover rate) in ten rat materials, including excreta, namely feces and urine, and organs, namely blood plasma, brain, muscle, heart, liver, spleen, lung and kidney was tested. The data were analysed with piecewise linear/non-linear exponential modelling that allows quantifying delay and turnover rate simultaneously. Additionally, effects on the whole body nitrogen balance and on organ specific growth were investigated.

Results

Nitrogen balance and growth

The dietary nitrogen uptake was 126 mg/d (95% confidence interval: 110 mg/d – 142 mg/d) during the last phase of the equilibration period. It remained identical in the 'low protein' group, but increased to 341 mg/d (333 mg/d – 349 mg/d) in the 'high protein' group after the start of the chase period (Fig. 9). The fecal nitrogen excretion was small and identical between both experimental groups and did not change during the whole chase phase. It was 16 mg/d (6 mg/d – 26 mg/d) and 21 mg/d (11 mg/d – 31 mg/d) in the low and high protein group, respectively. The renal nitrogen excretion was equally 63 mg/d (55 mg/d – 71 mg/d) for both experimental groups during the equilibration period, but increased exponentially to 280 mg/d (262 mg/d – 298 mg/d) in the 'high protein' group as a result of the higher protein intake during the chase period, while it remained at the initial value in the 'low protein' group. Hence, the nitrogen net balance was equal between experimental groups and significantly positive, indicating assimilation. Growth caused assimilation of 47 mg/d (33 mg/d – 61 mg/d) and 40 mg/d (26 mg/d – 54 mg/d) of nitrogen in the low and high protein group, respectively.

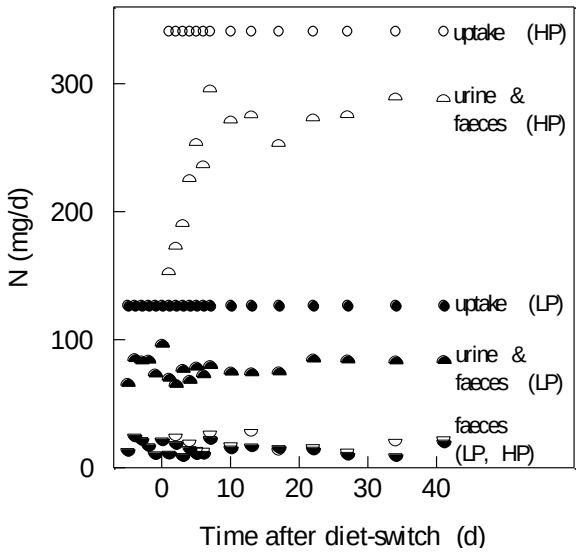

Figure 9: Dietary nitrogen uptake and nitrogen losses via fecal and renal excretion are shown for the ‚low' (closed symbols, abbreviated LP) and the ‚high protein' group (open symbols, HP). The difference between dietary nitrogen uptake and accumulated (renal and fecal) excretion is the net nitrogen balance. The time of the diet switch is time 0.

The animals' fresh weight increased from ~150 g to ~190 g ($y = 148e^{0.009x}$, $p < 0.001$, $r^2 = 0.73$) during the chase period, as a result of muscle tissue weight increasing from ~95 g to ~125 g ($y = 95e^{0.007x}$, $p < 0.001$, $r^2 = 0.78$ Fig. 10). The growth was better represented by an exponential model than by a linear model (RMSE was 4.0 and 4.9, respectively). The muscle growth rate constant of 0.007 d^{-1} indicated muscle growth of ~0.7 g d^{-1}. The growth rate of muscle and the growth rate of the whole body did not differ significantly. The other investigated organs did not change weight during the chase period (the growth rate obtained by regressing weight vs time was not significantly different from 0, but was significantly different from 0.007 d^{-1} and hence from 0.009 d^{-1}, which were the growth rates of muscle and whole body, respectively). Experimental groups did not differ in growth for none of the organs.

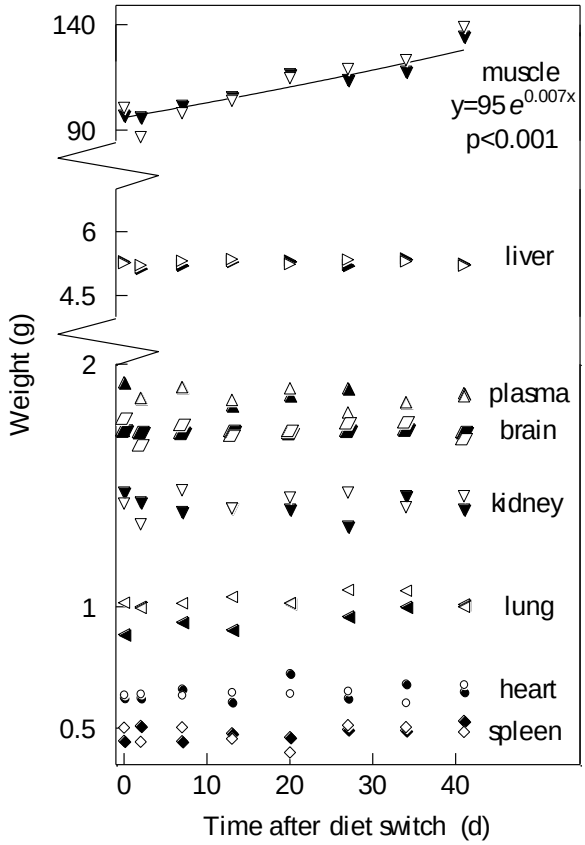

Figure 10: The organ specific fresh weights in the ‚low' (closed symbols) and the ‚high protein' group (open symbols) during the chase period. Muscle (combined weight of musculus femoris and musculus quadriceps) was the only organ that grew significantly. Please note the double broken y-axis.

Isotopic reaction progress

The initial isotopic values from day zero of the chase period were: blood plasma (-22.5 ‰ $\delta^{13}C$ value and 1531.5 ‰ $\delta^{15}N$ value), brain (-22.4 ‰ and 1433.5 ‰), heart (-21.4 ‰ and 1443.5 ‰), liver (-22.7 ‰ and 1635.4 ‰), lung (-22.1 ‰ and 1502.3 ‰), kidney (-21.7 ‰ and 1655 ‰), muscle (-21.5 ‰ and 1214 ‰) and spleen (-21.8 ‰ and 1516.0 ‰), urine (-21.1 ‰ and 1818.1 ‰) and feces (-25.0 ‰ and 1635 ‰). Their standard deviation was near identical and small among all organs and excreta (± 0.2 for $\delta^{13}C$ values and ± 34 for $\delta^{15}N$ values). Thus the coefficient of variation (i.e. standard deviation / mean) was identical and small among all organs and excreta (± 0.01 $\delta^{13}C$ values and ± 0.02 for $\delta^{15}N$ values), indicating that the labeling was practically equal among all rats.

The measurements at 2 d post diet-switch already exhibited a distinct reaction progress compared to the initial isotopic signatures. However, all accepted turnover models (i.e. models with parameters d, δ_∞ and r that produced minimum RMSE for a given organ or excrement) showed that the isotopic compositions of the organs and excreta did not react immediately after the diet-switch, but exhibited a delay (Fig. 11, inset). The delay was neither significantly different between ‘high protein’ and ‘low protein’ group, nor between carbon and nitrogen isotopes, nor between organs and excreta, but was highly significantly different from zero ($p < 0.001$, $n = 38$). The mean delay was 0.5 d (95% confidence interval: 0.2 – 0.7).

After the delay, the isotopic composition of all organs and excreta exponentially approached the isotopic composition of the unlabelled diet. Since most organs and excreta did not grow (weight gain was significant only for muscle, Fig. 10), Eqn 13 was applied to quantify half-lives, except for the growing muscle, to which the ‘mass model’ (Eqn 14) was applied to disentangle growth and turnover. For example, the isotopic incorporation in muscle had an apparent half-life of 30 d for carbon in the ‘low protein’ group (results from fitting Eqn 13). In contrast, the true half-life was 38 d when the exponential growth rate constant of 0.007 d^{-1} was considered (Eqn 14).

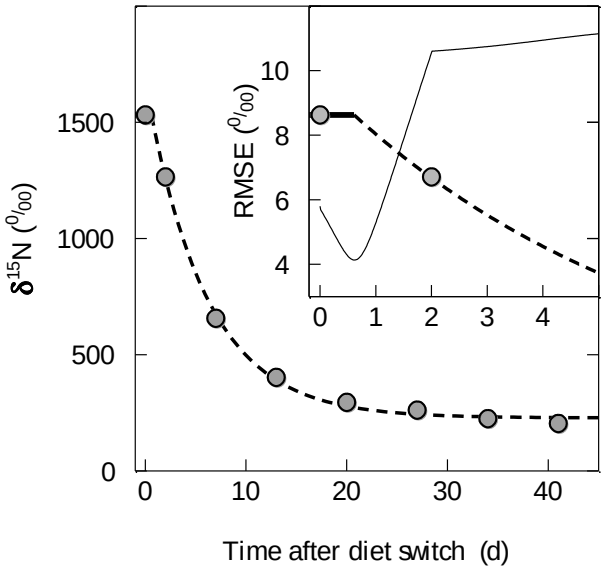

Figure 11: The reaction progress of $\delta^{15}N$ values for blood plasma (grey points) after an isotopic diet switch at time 0. The continuous horizontal line represents the delay and the following reaction progress is represented by the dashed line. The inset magnifies the first 5 d. The continuous thin line represents the RMSE for models differing in delay length.

The half-lives ranged from 1 d to 45 d (Table 8). This range was almost entirely caused by differences between different organs and different excreta. The order of organs and excreta regarding their half-lives was the same for carbon and nitrogen and it was the same for low and high protein groups, but the absolute half-lives differed significantly between carbon and nitrogen and between low and high protein groups.

The carbon and nitrogen half-lives were significantly shorter in the 'high protein group', in a paired t-test of all organs and excreta from the 'low protein group' and the 'high protein group' ($p < 0.01$, $n = 9$). This lead to the regression ($t_{½HP} = 0.8\ t_{½LP} - 0.8$, $r^2 = 0.98$, $p < 0.001$, $n = 18$) between half-lives of the 'low protein' and half-lives of the 'high protein' group independent of the element (Fig. 12). The slope of 0.8, which was significantly smaller than one, indicated that the absolute difference in half-lives between high- and low-protein-fed rats increased with increasing half-lives of the organs and excreta. The intercept, which was significantly larger than zero, indicated that half-lives for high-protein rats were additionally 0.8 d shorter than for low-protein rats, even for the

excreta with the shortest half-lives. Remarkably, this was also true for the growing muscle despite the identical growth rates of both groups (r/g was ~2.2 in the 'low protein' group and ~3.5 the 'high protein' group).

Table 8: Organ or excrement specific half-lives for carbon and nitrogen isotopes (C and N, respectively) with 95% confidence intervals. Estimates are rounded to the nearest whole number. Half-lives with asterisks are growth-corrected (using Eqn 14). Half-lives do not include the delay of ~0.5 d.

Organ or excrement	Element	Half-life (d)	
		'low protein'	'high protein'
Urine	N	4 (3 - 5)	1 (1 - 2)
Feces	C	2 (1 - 3)	1 (1 - 2)
	N	3 (2 - 4)	2 (1 - 2)
Plasma	C	5 (3 - 10)	3 (2 - 7)
	N	8 (7 - 8)	4 (3 - 4)
Liver	C	10 (6 - 18)	9 (4 - 29)
	N	10 (9 - 10)	6 (5 - 7)
Kidney	C	8 (3 - 17)	6 (3 - 12)
	N	10 (9 - 11)	7 (6 - 8)
Lung	C	9 (4 - 18)	8 (4 - 18)
	N	12 (9 - 16)	11 (9 - 13)
Spleen	C	7 (3 - 17)	6 (2 - 12)
	N	13 (11 - 14)	7 (5 - 9)
Brain	C	19 (11 - 55)	14 (5 - 35)
	N	21 (15 - 28)	16 (14 - 19)
Heart	C	30 (5 - 57)	25 (7 - 46)
	N	32 (28 - 40)	24 (21 - 25)
Muscle*	C	38 (21 - 42)	26 (15 - 48)
	N	45 (13 - 75)	37 (11 - 51)

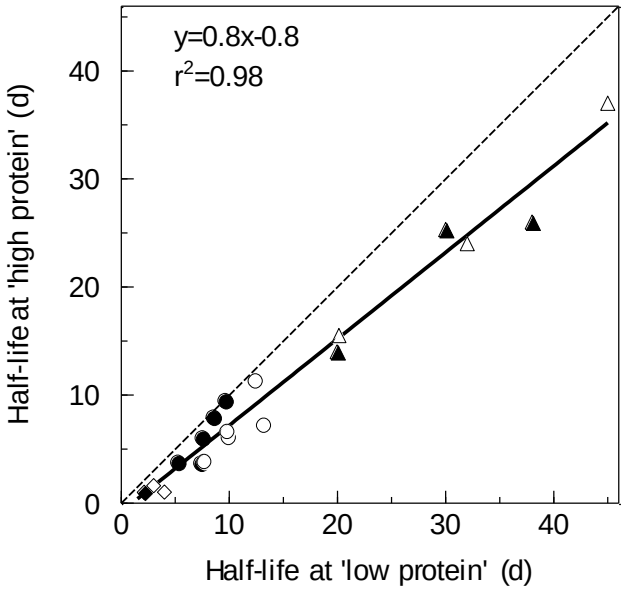

Figure 12: Correlation between specific half-lives of organs and excreta for rats fed low- and high-protein diets (note that carbon turnover was not quantified for urine). Triangles denote cerebral and muscular organs (brain, heart, muscle), circles denote visceral organs (plasma, liver, kidney, lung, spleen) and diamonds denote excreta (feces, urine). Black symbols denote nitrogen turnover, white symbols denote carbon turnover. The black line represents the regression line for all data. The 1:1 line is dashed. Half-lives do not include the delay of ~0.5 d.

In the ‘low protein’ group, the carbon half-lives were significantly shorter than the nitrogen half-lives in a paired t-test ($p < 0.05$, $n = 9$). The mean relative difference in half-lives was 31% ± 6% except for liver, brain and heart, where the relative difference was only 3% ± 2%. In the ‘high protein’ group, the carbon half-lives were still considerably shorter than the nitrogen half-lives by 18% ± 12%, except for liver and heart, but the difference was no longer significant ($p = 0.30$, $n = 9$).

The regression between carbon and nitrogen half-lives (Fig. 13) for the different organs and excreta was significant, independent of the experimental group ($r^2 = 0.93$, $p < 0.001$, $n = 18$) and closely following the 1:1 line indicating coupled carbon and nitrogen half-lives. The regression between carbon and nitrogen half-lives in the ‘low protein’ group did not differ significantly from that in the ‘high protein’ group, when considering the confidence intervals of slope and intercept.

Discussion

The effect of dietary protein content on the isotopic carbon and nitrogen turnover in ten organs and excreta obtained from rats was tested. The half-lives of both elements were significantly shorter, when rats were fed a diet with higher protein content (Fig. 12), whilst delay remained constant. These findings are based on the most complete set of organ specific isotopic turnover within one mammal, available at the present time The turnover rates presented herein can be considered 'real' turnover that were not obscured by increased isotopic incorporation due to growth, because the effects of growth and turnover were disentangled when necessary. The results indicated that isotopic diet reconstruction is confronted with variation in half-lives within the same organ or excrement, altering the time window through which information can be perceived, depending on dietary protein content. Such caveats may arise especially in omnivores with a broad trophic niche width, e.g. humans or bears whose diet may range from terrestrial plants to fish (Hilderbrand *et al*., 1996). Even in the diet of herbivores the protein content may differ by a factor of three, as the range examined in this study, due to changes in nitrogen supply and ontogenesis of the vegetation (Lemaire & Gastal, 1997).

The half-lives of the 'high protein' group were, on average, ~40% shorter compared to another study with rats, which fed a similar protein content, but neglected a delay in the exponential turnover models (Kurle, 2009). Since neglecting a delay overestimates the half-lives (Cerling *et al*., 2007; Zazzo *et al*., 2008), the differences in half-lives became insignificant when the delay was neglected in the models of this study (results not shown). This illustrates the importance of considering digestive transit time that implies a delay after a diet-switch. Further, this study showed the importance of organ-specific weight measurements, because the growth rate of the whole organism differed to the growth rates of the organs (Fig. 10), and adopting the growth rate of the whole organism for all organs would have obscured the results.

Influence of diet on delay and turnover rate

The mean delay was ~0.5 d and did not differ significantly between carbon and nitrogen isotopes; neither was it affected by the protein content of the diet. This is in line with the finding that the digestive transit time in rats is not substantially influenced by their dietary protein content (Raczynski *et al*., 1982). In contrast, these authors found an effect of dietary fibre content on the digestive transit time in rats. Whether or not the dietary fibre content also affects the delay in isotope turnover needs further investigation. The delay found here agreed with the lower boundary of digestive transit times, ranging from 0.4 to 3 d (Raczynski *et al*., 1982). This is reasonable as artificial feed lacking structure was used, and since gastric and small intestine transit times are ~1.5 h and ~3 h, respectively (Tuleu *et al*., 1999). This leaves the largest amount of the delay time (~10 h) for dietary nutrients to pass the gastro-intestinal barrier, become part of the systemic circulation and supply material for renewal.

Rats fed a diet with higher protein content had 30% shorter nitrogen half-lives (Table 8). The same trend appeared for carbon although it was less strong, with 20% shorter carbon half-lives, and the decrease in half-lives was not significant because of the lower detection limit due to the smaller labeling of carbon compared to nitrogen. The isotopic turnover as quantified herein is connected to other turnovers, like the turnovers of functional groups, amino acids, proteins or even cells. Likely, the faster nitrogen turnover not only resulted from a faster transamination, which would not affect the carbon turnover, but also indicates a faster protein turnover. The dietary protein content regulates the circulating amino acid concentration, which regulates the secretion of anabolic and catabolic hormones. This, in turn, regulates protein turnover, which is an important part of isotopic nitrogen turnover (Miron *et al*. 2006; Waterlow, 2006; Tsahar *et al*., 2007; Martinez del Rio *et al*., 2009). Since carbon turnover integrates protein, carbohydrate and fat turnover, either carbohydrate or fat turnover or both may have reacted differently to protein turnover and caused the weaker effect on carbon turnover. However, there were no clear differences between organs high in fat or carbohydrates (high fat content in brain or high glycogen content in liver) and the defatted muscle.

In line with increasing turnover rates, the renal nitrogen excretion increased in the high protein group, while it remained constant in the low-protein group (Fig. 9). The exponential increase indicated a retarded metabolic adaptation to the high protein diet. Since this was not apparent in the isotopic data, models assuming metabolic steady state (except for growth) were chosen, which might have lead to a small overestimation of half-lives.

Order of turnover rates

The order of organs and excreta regarding turnover rates was consistent for carbon and nitrogen isotopes and it was unaffected by the protein content of the diet. Three groups were evident, when considering the confidence intervals. The excreta were fastest; the visceral organs plasma, liver, kidney, lung and spleen were intermediate, whereas the cerebral and muscular organs brain, muscle and heart were slow (Table 8). This order is consistent with another rat study (Kurle, 2009), and with other rodents like house mouse (MacAvoy *et al*., 2005) and deer mouse (Miller *et al*., 2008) and even with non-mammals, like birds (Hobson & Clark, 1992; Bauchinger & McWilliams, 2009). This indicates that a general order of short, intermediate, and long isotope half-lives exists, independent of the study animal, its diet and environmental circumstances (Kurle, 2009). Visceral organs, which have a faster protein turnover (Waterlow, 2006; Martinez del Rio *et al*., 2009), have generally shorter half-lives than muscular and cerebral tissues. The large range of half-lives among the organs and excreta within one organism, which spans almost two orders of magnitude allows to establish 'isotopic clocks' (Guelinckx *et al*., 2008). The consistent order of organs and excreta among nutritional groups and among elements is advantageous. Such 'isotopick clocks' can be used where the changes in the diet of animals due to movement either have to be identified like for migrating wildlife or where they have to be excluded like in the case of authentication (Hobson & Clark, 1992; Bahar *et al*., 2009). The order of organs likely also holds true for much larger animals than rats, since the fractional rate of isotopic incorporation of specific organs should decline with body mass to approximately $-1/4^{th}$ the power (Martinez del Rio *et al*., 2009). According to this

relation and given the half-life of muscle in rats (~40 d), the body weight of rats (~200 g) and the body weight of a much larger animal, e.g. a cow (~500 kg), the half-life of muscle in a cow is predicted as ~150 d, which agrees with the results of a study that analyzed the isotopic turnover of muscle in cows (Bahar *et al*., 2008). The power law predicts that a weight ratio of 10^4 will cause a difference in turnover rate of one order of magnitude, while the turnover of organs and excreta within the rats almost covered two orders of magnitude (Fig. 13). The variation within one animal is thus larger than the variation between most animals differing in size.

Coupling of carbon and nitrogen turnover rates

This study revealed a strong correlation between carbon and nitrogen half-lives within each organ and feces, close to the 1:1 line (Fig. 13). This relation did not change significantly, when carbon and nitrogen half-lives of another 22 studies were added, including studies on fish (Guelinckx *et al*., 2007), reptiles (Seminoff *et al*., 2007), birds (Haramis *et al*., 2001) and mammals (Hilderbrand *et al*., 1996). It seems that this relation is independent of the animal class, including animals with poikilotherm and homoiotherm metabolism. This finding might contribute to the discussion as to whether or not carbon and nitrogen turnover are coupled or 'only' correlated (Carleton & Martinez del Rio, 2005; MacAvoy *et al*., 2005; Kurle, 2009).

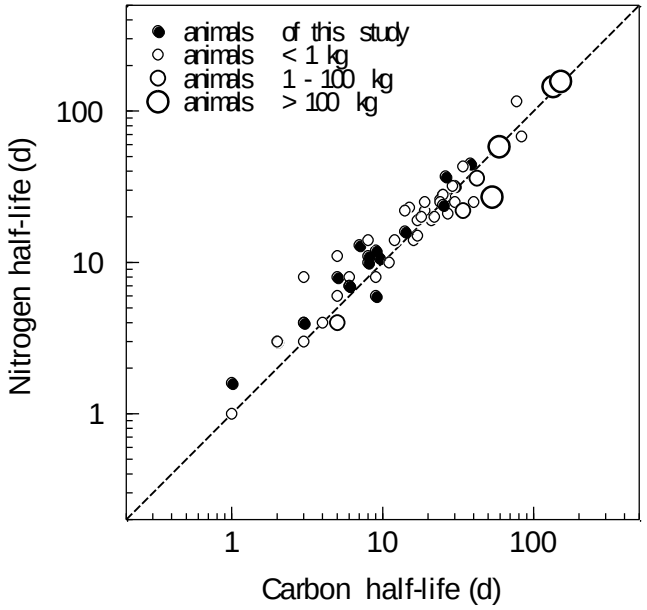

Figure 13: Correlation between carbon half-life and nitrogen half-life within the same organ or excreta from this study (n = 18) and from results obtained from 22 publications (n = 50). The publications comprise such divers organs as muscle from Sand Goby (Guelinckx *et al*., 2007), plasma and red blood cells from Black Bear (Hilderbrand *et al*., 1996), but also whole homogenized arthropods (Kaufman *et al*., 2008). The 1:1 line is dashed.

Conclusions

A statistical method to quantify delay and turnover rate simultaneously, using piecewise linear/non-linear exponential models was introduced. A delay was evident for all organs and excreta and neglecting the delay would have caused an overestimation of half-lives. Dietary protein content affected carbon and nitrogen half-lives, but not the delay. Rats fed a higher protein ration had 30% shorter nitrogen half-lives, whereas the carbon half-lives became shorter by only 20%. This indicated that the renewal time of organs and excreta is dependent on the dietary protein content.

Experiment 4:
Transamination reduces heterogeneity in isotopic nitrogen composition of amino acids in animals

Abstract

The analysis of isotopic nitrogen composition ($\delta^{15}N$) of amino acids allows detailed insights into trophic systems, because different amino acids carry different dietary information. However, a physiologically based explanation on the differing isotopic information of amino acids and knowledge on the time period through which the isotopic information is retained in organisms is missing. Here, it was hypothesized that the joint consideration of transamination and *de novo* synthesis divides amino acids into three functional groups that largely explain the dietary information contained in amino acids. Additionally, the isotopic turnover of amino acids that determines the time period through which information can be perceived was investigated.

Eighteen rats were fed a diet with differently ^{15}N-labeled amino acids. The redistribution of the dietary ^{15}N labels among the muscular amino acids was analyzed. Subsequently, the labelling was changed and the isotopic nitrogen turnover was analysed at amino acid level.

The amino acids had a common nitrogen half-life of ~20 d despite their differences in biochemical pathways. This indicated that their half-lives were determined by protein turnover. The $\delta^{15}N$ of amino acids differed according to their specific biochemical capacities for *de novo* synthesis and transamination: non-transaminating and essential amino acids remained relatively unaltered from diet to muscle tissue. Non-transaminating and non-essential amino acids showed an isotopic nitrogen composition that was between their dietary composition and that of their *de novo* synthesis pool, likely indicating their fraction of *de novo* synthesis.

Thus, non-transaminating and essential amino acids largely conserve the $\delta^{15}N$ of their primary producers and can be used to trace their origin in heterogeneous diets. In non-

transaminating but non-essential amino acids, the $\delta^{15}N$ can be used to quantify their fraction of *de novo* synthesis, while the bulk of amino acids, which are transaminating, share a common N pool, which likely contributed to the similarity of trophic shifts in food chains despite the dissimilarities among diets.

Introduction

The isotopic nitrogen composition ($\delta^{15}N$) of organismic materials provides information on trophic systems (Peterson & Fry 1987; Popp *et al.,* 2007). While the $\delta^{15}N$ of bulk materials indicates the trophic position of organisms (Minagawa & Wada 1984; Schmidt *et al*., 2004), the $\delta^{15}N$ of specific compounds has the potential to provide more detailed insights, because different compounds differ in biosynthetic pathways and thus may reveal isotopic information from different (dietary) sources (McMahon *et al*., 2010). This is particularly true for amino acids, as they provide the main nitrogen reservoir of bulk materials, but have individual biosynthetic pathways (Berg *et al*., 2010). Thus, alanine, aspartic acid and glutamic acid (among others) reflect the trophic position similar to bulk tissues, whereas glycine and phenylalanine provide an accurate determination of the $\delta^{15}N$ at the base of the food web (McClelland & Montoya 2002; Popp *et al*., 2007). However, reasons for this grouping as well as the information that can be obtained from other amino acids remain unknown. Understanding how the major biochemical mechanisms that govern nitrogen input and distribution, namely *de novo* synthesis and transamination, affect the $\delta^{15}N$ of amino acids would shed light on these knowledge gaps. Additionally, the time window through which such isotopic information in amino acids is retained in organisms and differences between amino acids in such time windows are worthy of investigation.

For carbon isotopic composition ($\delta^{13}C$), the amino acids' ability of *de novo* synthesis largely explained the information that can be retrieved from their $\delta^{13}C$ (McClelland & Montoya 2002; Popp *et al*., 2007). Essential amino acids remain biochemically unmodified from diet to consumer and thus represent the $\delta^{13}C$ of the amino

acids in the diet. For example, leucine (essential) was not enriched in ^{13}C between diet and consumer, whereas glutamic acid (non-essential) became enriched in ^{13}C. Remarkably, both amino acids were enriched in ^{15}N between diet and consumer (McClelland & Montoya 2002), indicating that the classification into essential / non-essential did not properly characterize the nitrogen isotopic information that are contained by amino acids. Apparently, a biochemical process that regulates nitrogen exchange beyond *de novo* synthesis and alters the $\delta^{15}N$ even of (at least some) essential amino acids interfered.

Transamination is the biochemical process of amino nitrogen transfer between an amino acid and an α-keto acid; in turn, the α-keto acid becomes an amino acid and vice versa (Berg *et al*., 2010). Since most transamination reactions have equilibrium constants close to one, the direction of a transamination reaction proceeds in large part as a function of the intracellular concentrations of the reactants (Mathews *et al*., 1999). Transaminations can also be chained, providing a "nitrogen shuttle" even between amino acids of different functional groups (e.g. essential and non-essential, Kalhan & Parimi, 2006). For example, the L-serine:glyoxylate aminotransferase (transamination enzyme, EC 2.6.1.45) catalyzes the reversible amino transfer from serine to glycine and the Glycine:2-oxoglutarate aminotransferase (EC 2.6.1.4) catalyzes the reversible amino transfer from glycine to glutamate. Glutamtate, as the central amino acid in metabolism (Berg *et al*., 2010), is again linked to other transamination chains, like the branched-chain amino acid metabolism that links the essential leucine, isoleucine and valine (Hutson *et al.*, 2005). Such chains provide continuous re-distribution of amino nitrogen among transaminating amino acids beyond *de novo* synthesis (Mathews *et al*., 1999). Hence, transaminating amino acids virtually share one common amino nitrogen pool (Hare *et al*., 1991). This should lead to a reduced heterogeneity in $\delta^{15}N$ among transaminating amino acids when the $\delta^{15}N$ of the diet is heterogeneous. However, since transamination fractionates, the $\delta^{15}N$ of transaminating amino acids is not completely equal (Macko *et al*., 1987).

Some amino acids differ in their ability to transaminate. In mammals, tyrosine can be degraded but not be synthesized by transamination, because the equivalent α-keto acid that would accept nitrogen and turn into tyrosine is missing (Devlin, 2010). Hence,

tyrosine can only be a nitrogen donor to the common nitrogen pool. The amino acids lysine, threonine (Devlin, 2010), proline (Murray *et al*., 2010) and phenylalanine (in absence of the dysfunction phenylkentonuria, Berg *et al*., 2010) do not transaminate at all. Due to their disability of bidirectional transamination, these amino acids are termed "non-transaminating" in this study.

Since many isotopic diet reconstruction studies focus on feeding generalists (e.g. Hare *et al*., 1991; Popp *et al*., 2007) and feeding generalists likely have an isotopically heterogeneous diet, it should be investigated how the $\delta^{15}N$ of isotopically heterogeneous amino acids will change from diet to consumer, after nitrogen redistribution by *de novo* synthesis and transamination. It can be hypothesized that the joint consideration of transamination and *de novo* synthesis largely explains the variation in $\delta^{15}N$ among amino acids in consumers. Since transamination is post *de novo* synthesis, its effect on $\delta^{15}N$ of amino acids should be of major priority and hence amino acids should divide into three groups: (i) transaminating amino acids should have a rather homogeneous $\delta^{15}N$ irrespective of their variation in the diet, because their amino nitrogen is exchanged with that of other transaminating amino acids (a common nitrogen pool), (ii) non-transaminating and essential amino acids should retain their $\delta^{15}N$ from diet, because nitrogen is not exchanged and (iii) non-transaminating and non-essential amino acids should have a $\delta^{15}N$ that ranges between that of their dietary $\delta^{15}N$ and that of the $\delta^{15}N$ of the nitrogen donor pool that supplies *de novo* synthesis.

As transamination governs nitrogen exchange, its exchange rate could determine the isotopic nitrogen turnover and thus the time window through which isotopic information can be perceived. In this case, the isotopic nitrogen turnover should differ between amino acids, in particular between transaminating and non-transaminating amino acids. However, the effect of transamination on isotopic nitrogen turnover could be superimposed by the continuous release of amino acids from protein turnover, in particular by the degradation of proteins by proteolysis. In that case, the isotopic nitrogen turnover should be rather similar among amino acids.

Eighteen rats were fed a diet with twelve ^{15}N labeled amino acids for 7 weeks and the $\delta^{15}N$ of amino acids in muscle tissue was analyzed. Subsequently, the $\delta^{15}N$ of the amino acids in the diet was switched to quantify their isotopic nitrogen turnover rate, denoted in half-life. This was necessary to evaluate potential differences in turnover rate among amino acids that could induce differences in $\delta^{15}N$ due to different levels of labelling. In the following, isotopic composition is dneoted as atom fraction ($x(^{15}N)$), because its use is preferred over the use of δ values for tracer and mixing calculations (Brenna *et al*., 1997; Coplen ,2011).

Results

$x(^{15}N)$ of amino acids at the end of the equilibration period

At the end of the equilibration period, the $x(^{15}N)$ of amino acids varied by about factor two in muscle tissue, ranging from 0.0067 $x(^{15}N)$ in tyrosine to 0.0158 $x(^{15}N)$ in phenylalanine. The $x(^{15}N)$ of dietary amino acids did not correlate significantly with the $x(^{15}N)$ of muscular amino acids ($p > 0.05$, $r^2 = 0.29$), but the following pattern was apparent (Fig. 14): (i) The transaminating amino acids alanine, glutamic acid, glycine, isoleucine, leucine, serine and valine had a rather common $x(^{15}N)$ of 0.0108 (SD 0.0007), which was not statistically different from the mean $x(^{15}N)$ of the diet (0.0101 ± 0.0001). Hence, their $x(^{15}N)$ had changed substantially, including depletion and enrichment in ^{15}N. The change ranged from -0.0211 in glycine to 0.0071 in serine. (ii) The $x(^{15}N)$ of non-transaminating and essential amino acids lysine, threonine and phenylalanine was quite similar in diet and muscle; hence they appeared on the 1:1 line. (iii) The non-transaminating and non-essential amino acids proline and tyrosine had a $x(^{15}N)$ between initial (dietary) $x(^{15}N)$ and the $x(^{15}N)$ of their specific nitrogen donor pool. The coefficient of variation was small (~ 2 %) among replicates for all amino acids, indicating equal labelling among rats.

Turnover of amino acid ^{15}N

The $x(^{15}N)$ of all amino acids exponentially approached the $x(^{15}N)$ of the unlabelled diet. The mean half-life was 20 d (13 d – 27 d) and half-lives were not significantly different among amino acids and they were not statistically different from the half-life of the entire muscle (Fig. 15). In particular, half-lives were not different between essential/non-essential and transaminating/non-transminating amino acids.

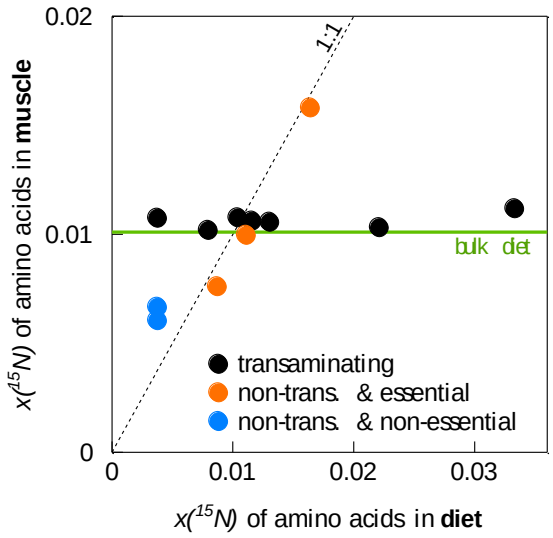

Figure 14: The atom fraction $x(^{15}N)$ of amino acids in diet and in muscle. Note the different ranges of both axes. "Non-trans." denotes non-transaminating amino acids. The 95 % confidence intervals had a similar size as the markers

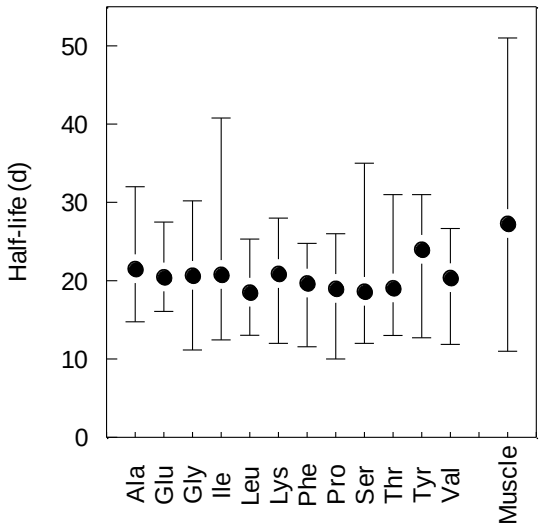

Figure 15: Half-lives of amino acids (quantified herein) and of muscle (quantified in Braun et al. 2013a), obtained from the same animals. Solid lines indicate the 95 % confidence intervals.

Discussion

It was hypothesized that the joint consideration of transamination and *de novo* synthesis divides amino acids into three groups that largely explain the variation in $x(^{15}N)$ among amino acids in animals: (i) transaminating, (ii) non-transaminating and essential, (iii) and non-transaminating and non-essential amino acids.

Transaminating amino acids had a rather homogeneous $x(^{15}N)$ value in consumer, despite their $x(^{15}N)$ value varied in the diet by an order of magnitude (Fig. 14). Exemplarily, serine was not provided by the labelled amino acid mixture, but had the same $x(^{15}N)$ value in the muscle tissue as the labelled amino acids (Table 4). The $x(^{15}N)$ value of transaminating amino acids was similar to the $x(^{15}N)$ of the bulk diet (plus an expectable isotopic enrichment, DeNiro & Epstein, 1981), and was similar to the mean of $x(^{15}N)$ of transaminating amino acids in the diet. This indicated rather complete redistribution of amino nitrogen among transaminating amino acids in consumer tissues that can be expected, because transamination is the most common reaction involving free amino acids (Devlin *et al*., 2010). However, the transaminations cause isotopic fractionation (Macko *et al*., 1986) that appears herein only as minor variation around the homogenized $x(^{15}N)$, because of the very high labelling. Future research should gain detailed knowledge on the isotopic fractionations of the specific transamination enzymes.

The homogenization of $x(^{15}N)$ of transaminating amino acids is quite consistent across trophic levels, environments and diets, as it can be found in herbivores, e.g. pig, and terrestrial and marine carnivores, e.g. lion and whale (see results of Hare *et al*., 1991). However, this finding is in contrast to $x(^{15}N)$ values in a zookplankter (McClelland & Montoya, 2002), indicating that the ability of transamination may differ between evolutionary more remote organisms that do not share conserved biochemical pathways. This calls for the need to repeat such investigations in other animal groups that are in the focus of isotopic animal ecology, e.g. birds.

Homogeneous $x(^{15}N)$ in consumer, but varying $x(^{15}N)$ in diet implies variation in trophic shifts among transaminating amino acids, including enrichment and depletion in

^{15}N, as found herein (Fig. 14) and in other studies (McMahon *et al*., 2010; Bloomfield *et al*., 2011). Amino acids become enriched in ^{15}N when their dietary $x(^{15}N)$ is below the homogenized $x(^{15}N)$ and vice versa; the height of the trophic shift is defined by the difference between isotopic composition of an amino acid and the homogenized $x(^{15}N)$. Hence, the trophic shift of individual transaminating amino acids may not indicate the trophic position of the consumer.

Non-transaminating and non-essential amino acids changed their $x(^{15}N)$ from diet to consumer to less extent. Considering their biochemical pathways, each amino acid of this group in consumer is either derived from its dietary amino acid pool (without nitrogen exchange by transamination) or synthesized *de novo* by the organism. Hence, the change in $x(^{15}N)$ should be a function of their dietary $x(^{15}N)$, the $x(^{15}N)$ of the donor pool supplying *de novo* synthesis, the fraction of *de novo* synthesized amino acids, and isotopic fractionation during *de novo* synthesis (Macko *et al.*, 1987): Principally, the $x(^{15}N)$ of these amino acids may retain their dietary $x(^{15}N)$ in case of no *de novo* synthesis (e.g. when dietary supply exceeds the need); the $x(^{15}N)$ of these amino acids should be equal to the $x(^{15}N)$ of the precursor pool (± isotopic fractionation) in case the fraction of *de novo* synthesis is 100 %; in cases where the fraction of *de novo* synthesis is between 0 and 100 %, the $x(^{15}N)$ of the amino acid should be between dietary $x(^{15}N)$ and $x(^{15}N)$ of *de novo* synthesis pool (± isotopic fractionation). When neglecting the relatively small isotopic fractionation compared to the high labeling herein, the *de novo* synthesis fraction as calculated by mass balance was 25 % for tyrosine and 40 % for proline, although their near identical $x(^{15}N)$ in consumer. This contrast resulted from differing nitrogen donor pools (tyrosine is supplied by phenylalanine, whereas proline is supplied by the common nitrogen pool, as glutamic acid is the precursor, Jones, 1985) that also differed in $x(^{15}N)$.

Non-transaminating and essential amino acids almost retained their dietary $x(^{15}N)$ value and hence provide highly conserved isotopic information from the base of the food web. However, minor changes in isotopic compositions might occur (e.g. small deviations from the 1:1 line in Fig. 14) due to isotopic fractionation, e.g. caused by the deamination of threonine (Hare *et al*., 1991).

The half-lives were rather constant among amino acids (Fig. 15), indicating that the differences in $x(^{15}N)$ values among amino acids were not influenced by differences in isotopic turnover rates that determine the kinetics of label incorporation. This also indicated that the common turnover was not an effect of transamination that differed between amino acids, but rather an effect of proteolysis that governs the degradation of whole proteins and thus affects nearly all amino acids at once. Amino acids had shorter half-lives than their bulk muscle tissue (Braun *et al*., 2013a), although statistically indistinguishable. This might result from the slower turnover of other nitrogen containing components in bulk muscle, like nucleic acids (Halvorson, 1958).

This study quantified the isotopic carbon turnover of 16 and the isotopic nitrogen turnover of 22 organismic materials obtained from a small monogastric animal (*Rattus norvegicus*) and from a large ruminant (*Bos taurus*), respectively. To the best of my knowledge, the following synopsis of isotopic turnover beyond species barriers pioneered new scientific territory allowing a more global understanding of isotopic turnover and its driving factors than the usual mono-organismic studies.

The turnover of all materials and isotopes had a delay of approx. 12 h and a reaction progress that was best fitted by a one-pool model, except for the material "whole milk", which was best fitted by a two-pool model. Carbon and nitrogen half-lives were rather similar within one material, but varied between materials by two orders of magnitude, from ~10 h to ~1000 h (Fig. 16). Half-lives were shortened by an increase in dietary protein content. Since the diet affects half-lives (among other factors), a procedure to validate isotopic turnover (i.e. delay and half-life) at differing (dietary) conditions was developed. By inverse modelling, the validation procedure could be turned into an estimation procedure allowing the quantification of isotopic turnover at natural conditions and thus overcoming the artificial conditions of diet-switch experiments. The compound specific analysis of twelve amino acids revealed a common turnover, but a specific isotopic nitrogen composition that was determined by the amino acids' abilities of transamination and of *de novo* synthesis.

No variation in delays

The delays of plasma and organs were equal. This indicated that the time after nutrients passed the gastro-intestinal barrier, i.e. transportation in systemic circulation and synthesis of precursors, is negligible for the delay. The delay should thus be determined by the digestion processes before.

Remarkably, the delays were also equal between organs of rat and cow, although the animals differ in digestive systems (monogaster vs polygaster). Whether the common delay is caused by one determining factor that is equal between digestive systems, e.g. the enzymatic breakdown rate in the stomach, or caused by several factors that differ between digestive systems (and animals), but compensate, e.g. rumination, passage distance, passage rate, remains to be investigated.

Substantial variation in half-lives

The half-lives of materials ranged from ~10 h to ~300 h in cow and from ~50 h to ~1000 h in rat (Fig. 16). The variation within an organism is likely caused by differences in protein turnover (Martinez del Rio *et al*., 2009). This study affirmed this assumption, because (i) the order of isotopic half-lives in rat, from excreta (feces and urine) to visceral organs (e.g. kidney) to cerebral and muscular organs (brain and muscle) was consistent with the order of protein turnover rates (Waterlow, 2006), (ii) protein and isotopic turnover are both affected by dietary protein content (see also Lobley, 2003), (iii) the half-lives of carbon and nitrogen within one material were rather similar. Proteins contain both elements and its turnover should thus affect the isotopic turnover of both elements consistently.

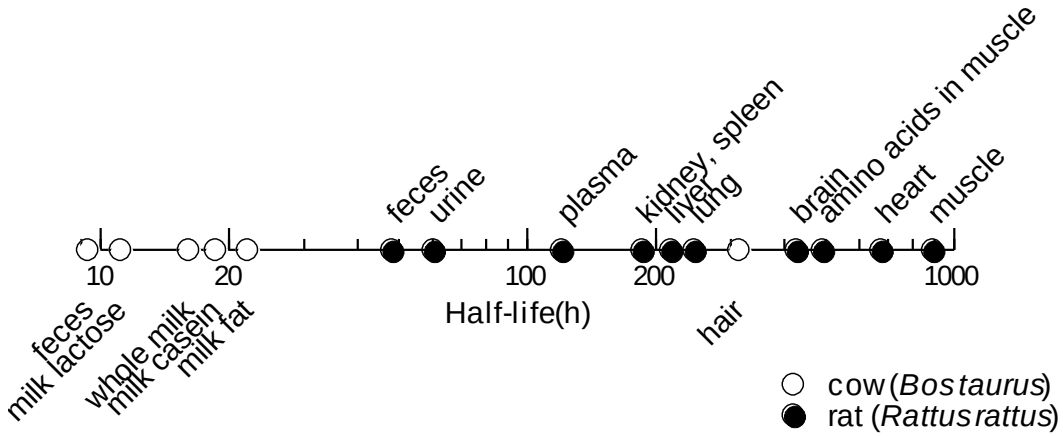

Figure 16: Half-lives of investigated materials. The mean half-life is shown when C and N half-lives have been quantified simultaneously in one material and when half-lives were quantified at differing dietary protein contents. Statistical uncertainty and variation between elements or variation caused by dietary protein contents was about the size of the markers.

Within the cow, the "apparent half-lives" of the excreta milk and hair were substantially shorter than the half-life of plasma (~ 700 h, Bahar *et al*., unpublished), although both excreta are synthesized from plasma constituents and should hence represent the plasma turnover. Since plasma is a heterogeneous organ, including dissolved proteins, glucose and free amino acids (Kratochwil *et al*., 2009), and since the synthesis of both excreta is mainly supplied by rather low-molecular constituents, e.g. glucose and amino acids, it can be hypothesized that the various plasma constituents turn over differently, where the low-molecular constituents turn over quicker than the high molecular compounds, e.g. dissolved proteins. Both excreta would thus represent the turnover of the low-molecular constituents in the plasma. This would imply that using the excreta as a proxy for (general) plasma turnover is improper. However, also the "apparent half-lives" of milk and hair differed by factor ~20, although (at least) milk casein and hair should be synthesized from the same plasma constituents, i.e. amino acids. Since the blood circulation in the tail tip (from which the hair was sampled) should be worse compared to the mammary glands in the odder (where milk is produced), the supply of constituents should be slower in the tail tip. The slower supply of constituents could cause the difference in "apparent half-lives". It remains to be tested whether the half-lives of hairs from better circulated areas are shorter than the half-lives of hairs from worse circulated areas, although principally supplied by the same pool.

Between organisms, the half-life of a specific organ declines with body mass to approximately $-1/4^{th}$ the power (hence named "size rule", Martinez del Rio *et al*., 2009). Thus, the half-lives of organs in rats allowed a rather precise prediction of the half-lives of organs in the cow (Fig. 17). Organ half-lives of small and easy-to-handle model organisms can thus be used to predict organ half-lives of bigger, wild and/or endangered species from which half-lives are often unknown. This could extent the application of the "isotopic clock" that uses the differing half-lives of two organs within an animal to predict the time of movement, metamorphosis or changing diets (Phillips & Eldridge, 2006).

However, the half-life of feces in the rat was *per se* longer than the half-life of feces in the cow – when considering the "size rule", this discrepancy was even aggravated (Fig. 17). This indicated that the "size rule" may be invalid to predict half-lives of excreta like

feces, whose constituents do not pass the gastro-intestinal barrier and thus never become part of the metabolism. Additionally, the half-life of feces in rat could have been influenced by caecotrophy, i.e. feeding on feces and thus recycling of nutrients. Knowing for which materials the "size rule" is valid needs further investigations.

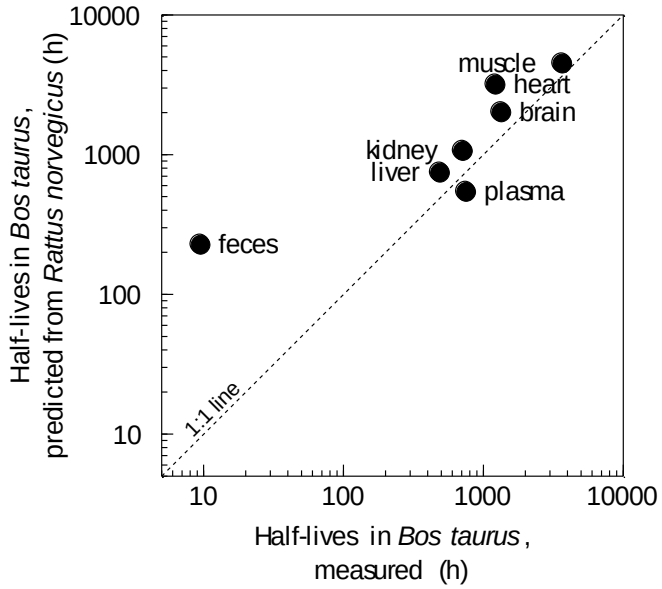

Figure 17: Half-lives of organs and excreta in *Bos taurus* as predicted from organs and excreta in *Rattus norvegicus*, according the "size rule" (Martinez del Rio *et al*., 2009). The half-lives of plasma, liver, kidney, heart and brain from *Bos taurus* are from Bahar *et al*. (unpublished). All other half-lives are from this study.

The disproportionate use and (miss-)interpretation of one-pool models

One-pool models have been predominantly used in turnover studies (e.g. herein, Fry & Arnold, 1982; Hobson & Clark, 1992; Podlesak *et al*., 2005), although more-pool models likely better characterize the complex interactions of elemental pools and sources (Martinez del Rio *et al*., 2009). Pragmatic reasons are: (i) one-pool models are more simple in modelling and interpretation (Martinez del Rio *et al*., 2009), (ii) the limited knowledge of the arrangement of an unknown but possibly large number of pools that may be in sequence, parallel or branched (Fig. 18, legend), but also (iii) analytical limitations may add: An ideal isotopic-switch experiment has error-free data, infinite short sampling intervals and the sampling lasts until complete turnover (among others). Each of five pool arrangements consisting of one or two pools produces then a clearly defined reaction progress (see solid lines in Fig. 18). The pool numbers can be disentangled in linear-scale (Fig. 18 A) by curve fitting assisted by a statistical information criterion, most importantly the AIC (Martinez del Rio & Anderson-Sprecher, 2008), or in log-scale (Fig. 18 B) using the "reaction progress variable method" (Cerling *et al*., 2007). However, when considering a typical measurement error of 0.5 ‰ (indicated by colored shades in Fig. 18) at a typical isotopic switch of 10 ‰, some reaction progresses overlap. The sequential two-pool system overlaps with the slow one-pool system and the branched two-pool system overlaps with the fast one-pool system at any time of the reaction progress (although adopting the kinetics of the slow one-pool system after complete turnover of the fast pool). The experimental error is usually considerably larger than the measurement error due to unavoidable variation in feed digestibility, isotopic and chemical composition; unavoidable variation in animal conditions like body size, feeding preferences and metabolic peculiarities like diseases or decreasing milk yield; unavoidable variation in environmental conditions like day and night or seasons; unavoidable variation in the sampled body part like the position within an organ, the chemical composition of an tissue. Considering the experimental error and following the principle of parsimony ("all other things being equal, the simplest solution is the best"), one-pool models may often seem reasonable for these two-pool systems.

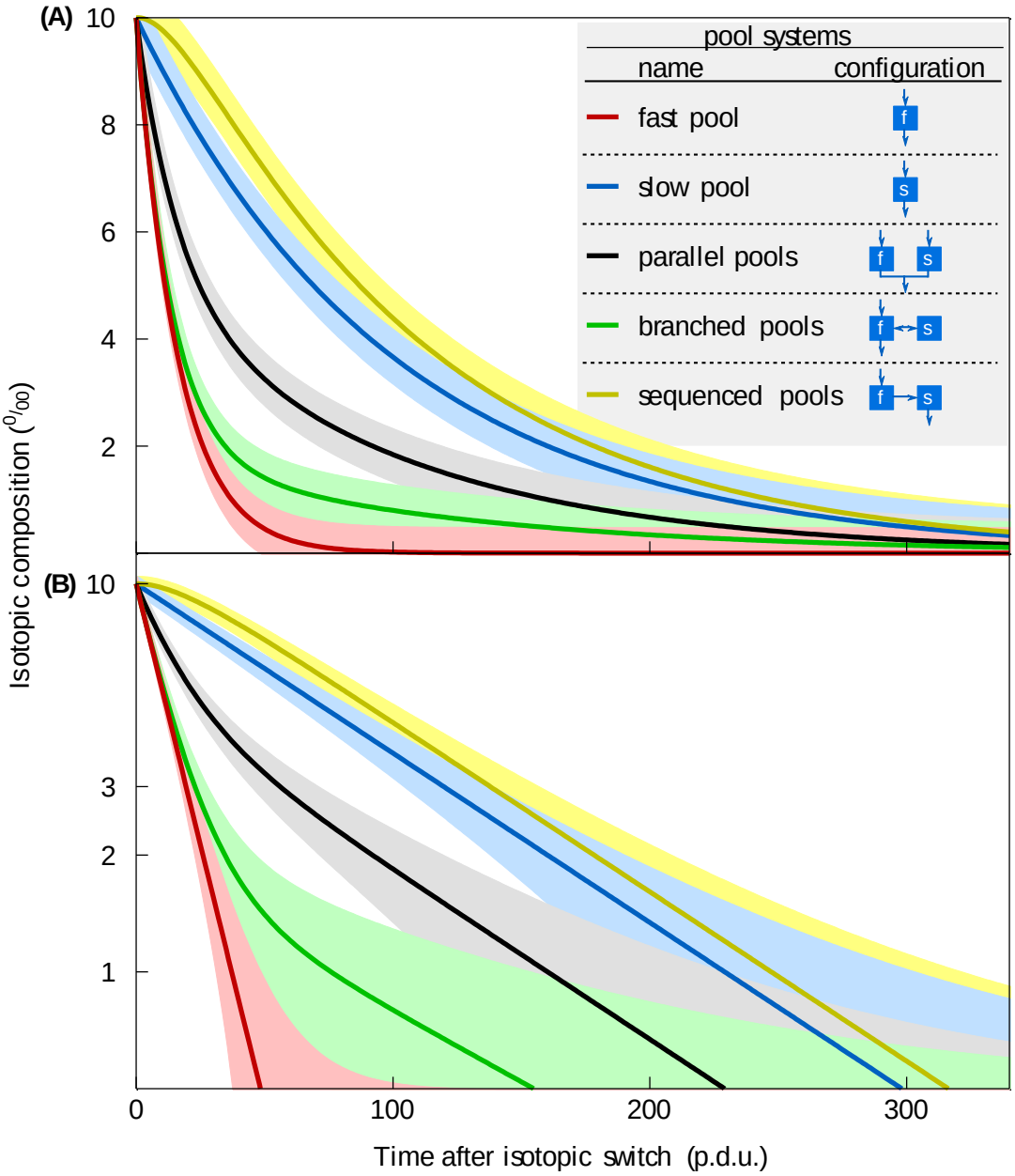

Figure 18: The reaction progress of a fast and slow one-pool system (half-lives differ by factor seven, as between plasma and muscle in rats, indicated by *f* and *s*, respectively) and systems consisting of two pools of equal size that are either parallel, branched or sequenced (same half-life as the fast and slow one-pool systems). Delays are not included. Shaded areas denote a typical measurement error of 0.5 ‰. The y-axes of panel (A) is in linear-scale, whereas the y-axes of panel (B) is in log-scale. P.d.u. indicates process defined units, as the time scale can be hours, days etc.

A misuse of one-pool models reveals several fundamental problems: (i) in case of a branched two-pool system, the sampling design rather than the pool system itself determines the one-pool modelling results. A (too) short sampling period that does not consider complete turnover overemphasizes the fast pool, whereas too wide sampling intervals (e.g. beyond turnover time of the fast pool) overemphasize the slow pool, especially when the fast pool is small. (ii) In case of a sequenced two-pool system, a one-pool model will mainly represent the half-life of the slow pool (see consistent reaction progress in Fig. 18), rather independent of sampling. The hesitant reaction progress after the isotopic switch might pretend a delay. Considering such "fake delays" will likely lead to biased half-lives. (iii) Remarkably, the reaction progress of a parallel two-pool system is distinct and can be disentangled statistically despite such erroneous data (also from a one-pool system with mean half-life, data not shown). However, parallel and thus rather independent body pools are hardly realistic – exceptions might be materials where compounds are mixed after synthesis, like milk.

In this study, a branched two-pool system is a realistic assumption for the turnover of amino acids in the muscle, because free amino acids enter and leave the muscle cells (fast "passage" pool) and exchange with the myofibrils (slow "storage" pool, Mente *et al*., 2002). The half-life of the passage pool is ~1 h (in humans, Waterlow, 2006). Since amino acids were first sampled 2 d after the isotopic-switch, the reaction progress of the passage pool must have been missed. Hence, the one-pool model likely quantified the half-life of the storage pool. In line, the estimated half-life is close to the half-life of proteins undergoing proteolysis in human skeletal muscle (Poortmans *et al*., 2012). Since the proteolysis of one protein simultaneously releases a complete set of amino acids, proteolysis should homogenize the amino acid turnover, which is in line with the result found herein.

The branched two-pool model is also reasonable for the other organs in the rat (Poupin *et al*., 2013). Since the sampling scheme was the same, it is likely that the storage pool of these materials was quantified. The bulk carbon / nitrogen turnover also include nucleic acids, which extended the half-life of muscle compared to the half-life of muscular amino acids, because deoxyribonucleic acids turn over rather slowly (Swick *et al*., 1956).

Whole milk consists of individual milk components (that are synthesized separately) and hence a parallel pool model can be assumed (at least in short-term). Since parallel pool systems can be disentangled rather easily despite error in the data (see above), a two-pool system represented the whole milk best and its half-lives were consistent with the half-lives of the individual milk components. For each milk component, an branched two-pool system is a rather realistic simplification, e.g. for fat it is known that fast passage pools (e.g. fat in blood plasma) exchange with slow storage pools (e.g. body fat stores, Matthews *et al*., 1956). Since the sampling interval was relatively short, but sampling did not cover complete turnover, it can be assumed that the fast passage pool was quantified for the milk components.

The measurement error of isotopic samples (~0.5 ‰) results from technical limitations of the mass spectrometry and should hence be rather constant. Increasing the height of an isotopic-switch would thus increase the signal-to-noise ratio and likely allow for a better differentiation between one- and more-pool systems. Such high isotopic-switches should be induced by switches of the isotopic composition at constant diet and not by diet-switches, because it was found that diet affected isotopic turnover. High isotopic switches alone are not satisfactory (as seen in the rat study, where the isotopic-switch was ~1500 ‰), but the sampling duration should consider complete material turnover and sampling intervals should be short enough to capture the effects of all pools. The latter might also help to disentangle "fake delays" from true delays. Since no sequenced pool systems have been assumed, all delays found herein can be considered true delays induced by digestion.

Conclusions

Three methods have been developed and applied to quantify the delay between ingestion and first reaction progress. Considering such a physiological essential should improve turnover estimation and hence its application.

Next to the pool assumptions also the sampling design affects and potentially biases the quantification and interpretation of delays and half-lives. However, a proper sampling design should consider complete turnover, sampling intervals that are shorter than the fastest pool and a high isotopic switch at else constant diet – properties that are practically hard to achieve at the same time. Thus, future hypothesis, sampling designs and data interpretation should focus on aspects, e.g. either the faster or the slower pools, of a (likely) complex pool system.

The synopsis of isotopic turnover beyond species barriers revealed that delays were independent of digestive systems and that half-lives of organs were predictable between organisms of different sizes, whereas the half-life of feces was not.

Acknowledgements

I am deeply grateful to Karl Auerswald for his never-ending guidance and support in scientific questions as well as for his open mind for new ideas and his critical but very comprehensive views on things that inspired countless discussions. Karl definitely helped me to expand my intellectual borders.

I thank Hans Schnyder for inspiring discussions and his critical view on my (sometimes) half-baked ideas and manuscripts that helped me to develop. In particular, I thank him for providing probably one of the best organized research facilities in the world.

I thank Jürgen Geist for mediating this PhD project, for always believing in me, supporting my ideas and for being a source of motivation. I also thank him for reviewing this PhD thesis, although he would likely prefer water related topics.

I thank Rudi Schäufele for conducting (nearly) all isotope measurements and for being my most loyal mensa-mate. I always enjoyed discussing with you about soccer, although you still have to learn that you support the wrong team.

I thank Stephan Schneider and Gerhard Bellof for conducting the cow experiment and for providing expertise in cow physiology.

Armin Vikari is thanked for email correspondence to rat related topics and for conducting the rat experiment.

I thank all employees of the "Lehrstuhl für Grünlandlehre", in particular Melitta and all other participants of the "morning coffee break".

Finally, I thank the DFG for granting this project [grant number AU 183/3-1].

To properly acknowledge the support of my parents, relatives, friends and life partner, the space herein would not be enough and hence I will thank them personally.

References

Alves-Stanley CD, Worthy GAJ (2009) Carbon and nitrogen stable isotope turnover rates and diet-tissue discrimination in Florida Manatees (*Trichechus manatus latirostris*). *Journal of Experimental Biology* 212: 2349.

Auerswald K, Wittmer M, Zazzo A, Schaeufele R, Schnyder H (2010) Biases in the analysis of stable isotope discrimination in food webs. *Journal of Applied Ecology 47:* 936.

Ayliffe LK, Cerling TE, Robinson T, West AG, Sponheimer M, et al. (2004) Turnover of carbon isotopes in tail hair and breath CO_2 of horses fed an isotopically varied diet. *Oecologia* 139: 11.

Bahar B, Schmidt O, Moloney AP, Scrimgeour CM, Begley IS, Monahan FJ (2008) Seasonal variation in the C, N and S stable isotope composition of retail organic and conventional Irish beef. *Food Chemistry* 106: 1299.

Bahar B, Moloney AP, Monahan FJ, Harrison SM, Zazzo A, et al. (2009) Turnover of carbon, nitrogen, and sulfur in bovine longissimus dorsi and psoas major muscles: Implications for isotopic authentication of meat. *Journal of Animal Science* 87: 905.

Bahar B, Harrison SM, Moloney AP, Schmidt O, Monahan FJ (unpublished) Isotopic turnover of C and N in bovine blood fractions and inner organs.

Barrett D (2002) Steady state turnover time of carbon in the Australian terrestrial biosphere. *Global Biogeochemical Cycles* 16: 55.

Bauchinger U, McWilliams SR (2009) Carbon turnover in tissues of a Passerine Bird: Allometry, isotopic clocks, and phenotypic flexibility in organ size. *Physiological and Biochemical Zoology* 82: 787.

Berclaz P Y, Benedek C, Jequier E, Schutz Y (1996) Changes in protein turnover and resting energy expenditure after treatment of malaria in Gambian children. *Pediatric Research* 39: 401.

Berg JM, Tymoczko JL, Stryer L (2010) Biochemistry. W. H. Freeman, New York.

Biolo G., Maggi SP, Williams BD, Tipton KD, Wolfe RR (1995) Increased rates of muscle protein turnover and amino acid transport after resistance exercise in humans. *American Journal of Physiology - Endocrinology and Metabolism* 268:514.

Bloomfield AL, Elsdon TS, Walther BD, Gier EJ, Gillanders BM (2011) Temperature and diet affect carbon and nitrogen isotopes of fish muscle: can amino acid nitrogen explain effects? *Journal of Experimental and Marine Biology and Ecology* 399: 48.

Boutton TW, Tyrrell HF, Patterson BW, Varga GA, Klein PD (1988) Carbon kinetics of milk formation in Holstein cows in late lactation. *Journal of Animal Science* 66: 2636.

Bowen GJ, Wassenaar LI, Hobson KA (2005) Global application of stable hydrogen and oxygen isotopes to wildlife forensics. *Oecologia* 143: 337.

Bowtell JL, Leese GP, Smith K, Watt PW, Nevill A, et al. (1998) Modulation of whole body protein metabolism, during and after exercise, by variation of dietary protein. *Journal of Applied Physiology* 1744: 52.

Braun A, Auerswald K, Vikari A, Schnyder H (2013a) Dietary protein content affects isotopic carbon and nitrogen turnover. *Rapid Communications in Mass Spectrometry* 27: 2676.

Braun A, Schneider S, Auerswald K, Bellof G, Schnyder H (2013b) Forward Modeling of Fluctuating Dietary 13C Signals to Validate 13C Turnover Models of Milk and Milk Components from a Diet-Switch Experiment. *PLoS ONE* 8(12): e85235.

Brenna JT, Corso TN, Tobias HJ, Caimi RJ (1997) High precision continuous-flow isotope ratio mass spectrometry. *Mass Spectrometry Reviews* 16: 227.

Carleton SA, Martinez del Rio C (2005) The effect of cold-induced increased metabolic rate on the rate of 13C and 15N incorporation in house sparrows (*Passer domesticus*). *Oecologia* 144: 226.

Carleton SA, Martinez del Rio C (2010) Growth and catabolism in isotopic incorporation: a new formulation and experimental data. *Functional Ecology* 24: 805.

Cerling TE, Harris JM, Leakey MG (1999) Browsing and grazing in elephants: the isotope record of modern and fossil proboscideans. *Oecologia* 120: 364.

Cerling TE, Ayliffe LK, Dearing MD, Ehleringer JR, Passey BH, et al. (2007) Determining biological tissue turnover using stable isotopes: the reaction progress variable. *Oecologia* 151: 175.

Chatterton NJ, Harrison PA, Bennett JH, Asay KH (1989) Carbohydrate partitioning in 185 accessions of *Gramineae* grown under warm and cool temperatures. *Journal of Plant Physiology* 134: 169.

Cheeseman JM (1986) Compartmental efflux analysis – an evaluation of the technique and its limitations. *Plant Physiology* 80: 1006.

Chamberlain PM, Bull ID, Black HI, Ineson P, Evershed RP (2006) The effect of diet on isotopic turnover in Collembola examined using the stable carbon isotopic compositions of lipids. *Soil Biology and Biochemistry* 38: 1146.

Codron D, Sponheimer M, Codron J, Newton I, Lanham J, et al. (2012) The confounding effects of source isotopic heterogeneity on consumer-diet and tissue-tissue stable isotope relationships. *Oecologia*, 169: 939.

Coplen TB (2011) Guidelines and recommended terms for expression of stable-isotope-ratio and gas-ratiomeasurement results. *Rapid Communications in Mass Spectrometry* 25: 2538.

DeNiro MJ, Epstein S (1978) Influence of diet on distribution of carbon isotopes in animals. *Geochimica et Cosmochimica Acta* 42: 495.

DeNiro MJ, Epstein S (1981) Influence of diet on the distribution of nitrogen isotopes in animals. *Geochimica et Cosmochimica Acta*. 37: 703.

Devlin M (2010) Textbook of Biochemistry with Clinical Correlations. John Wiley.

Ebert TA (1999) Plant and animal populations: Methods in demography. Academic Press, London.

Edwards JE, Huws SA, Kim EJ, Lee MRF, Kingston-Smith AH, et al. (2008) Advances in microbial ecosystem concepts and their consequences for ruminant agriculture. *Animal* 2: 653.

Eisenhart C (1947) The assumptions underlying the analysis of variance. *Biometrics* 3: 1.

Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon isotope discrimination and photosynthesis. *Annual Review of Plant Physiology and Plant Molecular Biology* 40: 503.

Fearon K, Hansell DT, Preston T, Plumb JA, Davies J, et al. (1988). Influence of whole-body protein-turnover rate on resting energy-expenditure in patients with cancer. *Cancer Research* 48: 2590.

Friedman AN (2004) High-protein diets: potential effects on the kidney in renal health and disease. *American Journal of Kidney Diseases* 44: 950.

Fry B, Arnold C (1982) Rapid C13/C12 turnover during growth of Brown Shrimp (*Penaeus Aztecus*). *Oecologia* 54: 200.

Gaine PC, Pikosky MA, Martin WF, Bolster DR, Maresh CM, et al. (2006) Level of dietary protein impacts whole body protein turnover in trained males at rest. *Metabolism* 55: 501.

Garlick PJ, McNurlan MA, Essen P, Wernerman J (1994) Measurement of tissue protein synthesis rates in vivo: a critical analysis of contrasting methods. *American Journal of Physiology - Endocrinology and Metabolism* 266:287.

Gessler A, Tcherkez G, Peuke AD, Ghashghaie J, Farquhar GD (2008) Experimental evidence for diel variations of the carbon isotope composition in leaf, stem and phloem sap organic matter in *Ricinus communis*. *Plant Cell and Environment* 31: 941.

Guelinckx, J, Maes P, Van Den Driessche B, Geysen F, Dehairs F, et al. (2007) Changes in delta 13C and delta 15N in different tissues of juvenile sand goby *Pomatoschistus minutus*: a laboratory diet-switch experiment. *Marine Ecology Progress Series* 341: 205.

Guelinckx J, Maes J, Geysen B, Ollevier F (2008) Estuarine recruitment of a marine goby reconstructed with an isotopic clock. *Oecologia* 157: 41.

Halvorson H (1958) Intracellular protein and nucleic acid turnover in resting yeast cells. *Biochimica et Biophysica Acta* 27: 255.

Hao Y, Auerswald K, Bai Y, Wittmer M, Schnyder H (2011) Variation in carbon isotope discrimination in *Cleistogenes squarrosa (Trin.)* Keng: patterns and drivers at tiller, local, catchment, and regional scales. *Journal of Experimental Botany* 62: 4143.

Haramis GM, Jorde DG, Macko SA, Walker JL (2001) Stable isotope analysis of Canvasback winter diet in upper Chesapeake Bay. *Auk* 118: 1008.

Hare PE, Fogel ML, Stafford Jr. TW, Mitchell AD, Hoering TC (1991) The isotopic composition of carbon and nitrogen in individual amino acids isolated from modern and fossil proteins. *Journal of Archaeological Science* 18: 277.

Hilderbrand GV, Farley SD, Robbins CT, Hanley TA, Titus K, et al. (1996) Use of stable isotopes to determine diets of living and extinct bears. *Canadian Journal of Zoology* 74: 2080.

Hobson KA, Clark RG (1992) Assessing avian diets using stable isotopes.1. Turnover of C13 in tissues. *Condor* 94: 18.

Hopwood AJ, Mannucci A, Sullivan KM (1996) DNA typing from human feces. *International journal of legal medicine* 108, 237.

Hutson SM, Sweatt AJ, LaNoue KF (2005) Branched-chain amino acids metabolism: implications for establishing safe intakes. *Journal of Nutrition* 135: 1557.

Johnson HA, Baldwin RL, France J, Calvert CC (1999) A model of whole-body protein turnover based on leucine kinetics in rodents. *Journal of Nutrition* 129: 728.

Jones ME (1985) Conversion of glutamate to ornithine and proline: Pyrroline-5-carboxylate, a possible modulator of arginine requirements. *Journal of Nutrition* 115: 509.

Kalhan SC, Parimi PS (2006) Transamination of leucine and nitrogen accretion in human pregnancy and the newborn infant. *Journal of Nutrition* 136: 2815.

Kaufman MR, Gradinger RR, Bluhm BA, O`Brien DM (2008) Using stable isotopes to assess carbon and nitrogen turnover in the Arctic sympagic amphipod *Onisimus Litoralis*. *Oecologia 158:* 11.

Kleiber M, Smith AH, Black AL (1952) Carbonate as precursor of milk constituents in the intact dairy cow. *Journal of Biological Chemistry* 195: 707.

Klein PD, Boutton TW, Hachey DL, Irving CS, Wong WW (1986) The use of stable isotopes in metabolism studies. *Journal of Animal Science* 63: 102.

Knobbe N, Vogl J, Pritzkow W, Panne U, Fry H, et al. (2006) C and N stable isotope variation in urine and milk of cattle depending on the diet. *Analytical and Bioanalytical Chemistry* 386: 104.

Kratochwil A, Scheibe R, Wieczorek H, Campbell NA, Reece JB, et al. (2009) Biologie – Der neue Campbell. Pearson, 8th edition.

Krömker V (2006) Kurzes Lehrbuch Milchkunde und Milchhygiene. Parey Bei M, 1st edition.

Kuha J (2004) AIC and BIC - Comparisons of assumptions and performance. *Sociological Methods & Research* 33: 188.

Kurle CM (2009) Interpreting temporal variation in omnivore foraging ecology via stable isotope modelling. *Functional Ecology* 23: 733.

Lattanzi FA, Ostler U, Wild M, Morvan-Bertrand A, Decau M-L, et al. (2012) Fluxes in central carbohydrate metabolism of source leaves in a fructan-storing C-3 grass: rapid turnover and futile cycling of sucrose in continuous light under contrasted nitrogen nutrition status. *Journal of Experimental Botany* 63: 2363.

Layman CA, Araujo MS, Boucek R, Hammerschlag-Peyer CM, Harrison E, et al. (2012) Applying stable isotopes to examine food-web structure: an overview of analytical tools. *Biological Reviews* 87: 545.

Lecomte N, Ahlstrom O, Ehrich D, Fuglei E, Ims RA, et al. (2011) Intrapopulation variability shaping isotope discrimination and turnover: Experimental evidence in Arctic Foxes. *PLoS One* 6: e21357.

Lehmeier CA, Lattanzi FA, Schäufele R, Wild M, Schnyder H (2008) Root and shoot respiration of perennial ryegrass are supplied by the same substrate pools: assessment by dynamic 13C labeling and compartmental analysis of tracer kinetics. *Plant Physiology* 148: 1148.

Lemaire G, Gastal F (1997) Nitrogen uptake and distribution in plant canopies. in *Diagnosis of the nitrogen status in crops* (Eds. G. Lemaire), Springer.

Lobley GE (2003) Protein turnover-what does it mean for animal production. *Canadian Journal of Animal Science* 83*:* 327.

Lobo, I (2008) Biological complexity and integrative levels of organization. *Nature Education* 1:141.

Loeffler K, Gäbel G (2013) Anatomie und Physiologie der Haustiere. UTB, Stuttgart 13th edition.

MacAvoy SE, Macko SA, Arneson LS (2005) Growth versus metabolic tissue replacement in mouse tissues determined by stable carbon and nitrogen isotope analysis. *Canadian Journal of Zoology* 83: 631.

Macko SA, Fogel ML, Engel MH, Hare PE (1986) Kinetic fractionation of stable nitrogen isotopes during amino acid transamination. *Geochimica et Cosmochimica Acta* 50: 2143.

Macko SA, Fogel ML, Hare PE, Hoering TC (1987) Isotopic fractionation of nitrogen and carbon in the synthesis of amino acids by microorganisms. *Chemical Geology* 65: 79.

Martin O, Sauvant D (2007) Dynamic model of the lactating dairy cow metabolism. *Animal* 1: 1143.

Martineau A, Lecavalier L, Falardeau P, Chiasson JL (1985) Simultaneous determination of glucose-turnover, alanine turnover and gluconeogenesis in human using a double stable-isotope-labeled tracer infusion and gas-chromatography mass-spectrometry analysis. *Analytical Biochemistry* 151: 495.

Martinez del Rio C, Anderson-Sprecher R (2008). Beyond the reaction progress variable: the meaning and significance of isotopic incoporation data. *Oecologia* 156: 765.

Martínez del Rio C, Wolf N, Carleton SA, Gannes LZ (2009) Isotopic ecology ten years after a call for more laboratory experiments. *Biological Reviews* 84: 91.

Martínez del Rio C, Carleton SA (2012) How fast and how faithful: the dynamics of isotopic incorporation into animal tissues. *Journal of Mammalogy* 93: 353.

Mathews CK, van Holde KE, Ahern KG (1999) Biochemistry. Prentice Hall.

Matthews LW, Spector S, Lemm J, Olynyk P (1956) Utilization of exogenous fat and turnover rate of endogenous fat in the normal and hypophysectomized rat. *American Journal of Physiology* 188: 308.

McClelland JW, Montoya JP (2002) Trophic relationships and the nitrogen isotopic composition of amino acids in plankton. *Ecology*. 83: 2173.

McMahon KW, Fogel ML, Elsdon TS, Thorrold SR (2010) Carbon isotope fractionation of amino acids in fish muscle reflects biosynthesis and isotopic routing from dietary protein. *Journal of Animal Ecology* 79: 1132.

Mente E, Coutteau P, Houlihan D, Davidson I, Sorgeloos P (2002) Protein turnover, amino acid profile and amino acid flux in juvenile shrimp *Litopenaeus vannamei*: effects of dietary protein source. *Journal of Experimental Biology* 205:3107.

Merriam-Webster's Collegiate Dictionary (2005) Springfield, MA: Merriam-Webster, 11th ed.

Miller JF, Millar JS, Longstaffe FJ (2008) Carbon- and nitrogen-isotope tissue-diet discrimination and turnover rates in deer mice, *Peromyscus maniculatus*. *Canadian Journal of Zoology 86:* 685.

Minagawa M, Wada E (1984) Stepwise enrichment of 15N along food chains: further evidence and the relation between 15N and animal age. *Geochimica and Cosmochimica Acta* 48: 1135.

Miron MLL, Herrera MLG, Ramirez PN, Hobson KA (2006) Effect of diet quality on carbon and nitrogen turnover and isotopic discrimination in blood of a New World nectarivorous bat. *Journal of Experimental Biology* 209: 541.

Murray RK, Granner DK, Mayes PA, Rodwell VW (2010) Harper's Illustrated Biochemistry. McGraw-Hill Medical.

Nocek JE, Tamminga S (1991) Site of digestion of starch in the gastrointestinal tract of dairy cows and its effect on milk yield and composition. *Journal of Dairy Science* 74: 3598.

Pannemans DL, Halliday D, Westerterp KR, Kester AD (1995) Effect of variable protein intake on whole body protein turnover in young men and women. *American Journal of Clinical Nutrition* 61: 69.

Passey BH, Cerling TE (2002) Tooth enamel mineralization in ungulates: Implications for recovering a primary isotopic time-series. *Geochimica et Cosmochimica Acta* 66: 3225.

Pearson SF, Levey DJ, Greenberg CH, del Rio CM (2003) Effects of elemental composition on the incorporation of dietary nitrogen and carbon isotopic signatures in an omnivorous songbird. *Oecologia* 135: 516.

Peterson BJ, Fry B Stable isotopes in ecosystem studies (1987). *Annual Review of Ecology and Systematics* 18: 293.

Piccini C, Marchetti A, Francaviglia R (2014) Estimation of soil organic matter by geostatistical methods: Use of auxiliary information in agricultural and environmental assessment. *Ecological Indicators* 36: 301.

Phillips DL, Eldridge PM (2006) Estimating the timing of diet shifts using stable isotopes. *Oecologia* 147: 195.

Pinheiro J, Bates D, DebRoy S, Sarkar D and the R Development Core Team (2012) nlme: Linear and Nonlinear Mixed Effects Models. R package version 3.1-104.

Podlesak DW, McWilliams SR, Hatch KA (2005) Stable Isotopes in breath, blood, feces and feathers can indicate intra-individual changes in the diet of migratory songbirds. *Oecologia* 142:501.

Poortmans JR, Carpentier A, Pereira-Lancha LO, Lancha Jr A (2012) Protein turnover, amino acids requirements and recommendations for athletes and active populations. *Brazilian Journal of Medical and Biological Research* 2012, 45: 875.

Popp BN, Graham BS, Olson RJ, Hannides CCS, Lott MJ, et al. (2007) Insight into the trophic ecology of yellowfin tuna, *Thunnus albacares*, from compound-specific nitrogen isotope analysis of pronteinaceous amino acids. In: Stable Isotopes as Indicators of Ecological Change, Dawson TE, Siegwolf RTW (editors).

Poupin N, Huneau JF, Mariotti F, Tomé D, Bos C, et al. (2013) Isotopic and modeling investigation of long-term protein turnover in rat tissues. *American Journal of Physiology - Regulatory, Integrative and Comparative Physiology* 304:218.

Raczynski G, Eggum BO, Chwalibog A (1982) The effect of dietary composition on transit-time in rats. *Zeitschrift für Tierphysiologie und Tierernährung* 47: 160.

R Core Team (2013) R: A language and environment for statistical computing. R Foundation for Statistical Computing, Vienna, Austria.

Ritter A, Hupet F, Muñoz-Carpena R, Lambot S, Vanclooster M (2003) Using inverse methods for estimating soil hydraulic properties from field data as an alternative to direct methods. *Agricultural Water Management* 59: 77.

Russell JB, Rychlik JL (2001) Factors that alter rumen microbial ecology. *Science* 292: 1119.

Schmidt K, McClelland JW, Mente E, Montoya JP, Atkinson A, et al. (2004) Trophic-level interpretation based on $\delta^{15}N$ values: implications of tissue-specific fractionation and amino acid composition. *Marine Ecology Progress Series* 266: 43.

Schneider S, Auerswald K, Schnyder H, Bellof G (2011) Suitability of eight techniques for estimating digestibility of herbage from continuously grazed all-day pasture. *Animal Feed Science and Technology* 170: 1.

Schnyder H, Schwertl M, Auerswald K, Schäufele R (2006) Hair of grazing cattle provides an integrated measure of the effects of site conditions and interannual weather variability on 13C of temperate humid grassland. *Global Change Biology* 12: 1315.

Schnyder H, Ostler U, Lehmeier C, Wild M, Morvan-Bertrand A, et al. (2012) Tracing carbon fluxes: Resolving complexity using isotopes. In Matyssek et al., editors. Growth and Defence in Plants, Ecological studies.

van Soest PJ (1994) Nutritional ecology of the ruminant, Second Edition. Cornell University Press.

Schulze ED, Turner NC, Nicolle D. Schumacher J (2006) Species differences in carbon isotope ratios, specific leaf area and nitrogen concentrations in leaves of Eucalyptus growing in a common garden compared with along an aridity gradient. *Physiologia Plantarum* 127: 343.

Schwarcz HP (1991) Some theoretical aspects of isotope paleodiet studies. *Journal of Archaeological Science* 18: 261.

Schwertl M, Auerswald K, Schnyder H (2003) Reconstruction of the isotopic history of animal diets by hair segmental analysis. Rapid Communications in Mass Spectrometry 17: 1312.

Seminoff JA, Bjorndal KA, Bolten AB (2007) Stable carbon and nitrogen isotope discrimination and turnover in pond sliders *Trachemys scripta*: Insights for trophic study of freshwater turtles. *Copeia* 3: 534.

Spalding KL, Arner E, Westermark PO, Bernard S, Buchholz BA, et al. (2008) Dynamics of fat cell turnover in humans. *Nature* 453: 783.

Susmel P, Spanghero M, Stefanon B, Mills CR, Cargnelutti C (1991) Effect of NDF concentration and physical form of Fescue hay on rumen degradability, intake and rumen turn-over of cows. *Animal Production* 53: 305.

Sponheimer M, Robinson TF, Cerling TE, Tegland L, Roeder BL, et al. (2006) Turnover of stable carbon isotopes in the muscle, liver, and breath CO2 of alpacas (*Lama pacos*). *Rapid Communications in Mass Spectrometry* 20: 1395.

Swick RW, Koch AL, Handa DT (1956) The measurement of nucleic acid turnover in rat liver. *Archives of biochemistry and biophysics* 68:226.

Tarnopolsky MA, McDougall JD, Atkinson SA (1988). Influence of protein intake and training status on nitrogen balance and lean body mass. *Journal of Applied Physiology* 64: 187.

Tcherkez G, Mahe A, Hodges M (2011) C-12/C-13 fractionations in plant primary metabolism. *Trends in Plant Science* 16: 499.

Trueman CN, MacKenzie KM, Palmer MR (2012) Identifying migrations in marine fishes through stable-isotope analysis. *Journal of Fish Biology* 81: 826.

Tsahar E, Wolf N, Izhaki I, Arad Z, Martinez del Rio C (2007). Dietary protein influences the rate of N-15 incorporation in blood cells and plasma of Yellow-vented Bulbuls (*Pycnonotus xanthopygos*). *Journal of Experimental Biology* 211: 459.

Tuleu C, Andrieux C, Boy P, Chaumeil JC (1999) Gastrointestinal transit of pellets in rats: effect of size and density. *International Journal of Pharmaceutics* 180: 123.

Ungar ED, Ravid N (1999) Bite horizons and dimensions for cattle grazing herbage to high levels of depletion. *Grass and Forage Science* 54: 357.

Voigt CC, Matt F, Michener R, Kunz TH (2003) Low turnover rates of carbon isotopes in tissues of two nectar-feeding bat species. *Journal of Experimental Biology* 206: 1419.

Waterlow JC (1984) Protein turnover with special reference to man. *Journal of Experimental Physiology 69:*409.

Waterlow JC (2006) Protein Turnover. CABI, Oxfordshire.

Wilson JR (1994) Cell-wall characteristics in relation to forage digestion by ruminants. *Journal of Agricultural Science* 122: 173.

Wingfield WE, Raffe MR (2002) The Veterinary ICU Book. Teton NewMedia.

Witting DA, Chambers RC, Bosley KL, Wainright SC (2004) Experimental evaluation of ontogenetic diet transitions in summer flounder (*Paralichthys dentatus*), using stable isotopes as diet tracers. *Canadian Journal of Fisheries and Aquatic Sciences.* 61: 2069.

Wittmer MHOM, Auerswald K, Tungalag R, Bai YF, Schaeufele R, et al. (2008) Carbon isotope discrimination of C3 vegetation in Central Asian grassland as related to long-term and short-term precipitation patterns. *Biogeosciences* 5: 913.

Wittmer MHOM, Auerswald K, Bai Y, Schaeufele R, Schnyder H (2010) Changes in the abundance of C3/C4 species of Inner Mongolia grassland: evidence from isotopic composition of soil and vegetation. *Global Change Biology* 16: 605.

Woodland RJ, Rodriguez MA, Magnan P, Glemet H, Cabana G (2012) Incorporating temporally dynamic baselines in isotopic mixing models. *Ecology* 93: 131.

Woodward SJR (1998) Bite mechanics of cattle and sheep grazing grass-dominant swards. *Applied Animal Behaviour Science* 56: 203.

Wright LE (2012) Immigration to Tikal, Guatemala: Evidence from stable strontium and oxygen isotopes. *Journal of Anthropological Archaeology* 31: 334.

Zazzo A, Harrison SM, Bahar B, Moloney AP, Monahan FJ, et al. (2007) Experimental determination of dietary carbon turnover in bovine hair and hoof. *Canadian Journal of Zoology* 85: 1239.

Zazzo A, Moloney AP, Monahan FJ, Scrimgeour CM, Schmidt O (2008) Effect of age and food intake on dietary carbon turnover recorded in sheep wool. *Rapid Communications in Mass Spectrometry* 22: 2937.

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Curriculum vitae

11/2010 – 2/2014	Technische Universität München (PhD-Thesis at Lehrstuhl für Grünlandlehre)
4/2009 – 10/2010	Technische Universität München (M.Sc. in Biology)
10/2005 – 4/2009	Universität Innsbruck (B.Sc. in Biology)
9/1996 – 7/2005	Staffelsee-Gymnasium Murnau
9/1992 – 7/1996	Grundschule Schlehdorf - Großweil
March 12th, 1986	*