The Journal of Experimental Biology 211, 2233-2238
Published by The Company of Biologists 2008
doi:10.1242/jeb.018523

Stable carbon isotopes in exhaled breath as tracers for dietary information in birds and mammals

Christian C. Voigt[1,*], Leonie Baier[2], John R. Speakman[3] and Björn M. Siemers[2]

[1]Evolutionary Ecology Research Group, Leibniz Institute for Zoo and Wildlife Research, Alfred-Kowalke-Straße 17, D-10315 Berlin, Germany, [2]Max-Planck-Institute for Ornithology, Sensory Ecology Group, Eberhard-Gwinner-Straße, 82319 Seewiesen, Germany and [3]Aberdeen Centre for Energy Regulation and Obesity, School of Biological Sciences, University of Aberdeen, Tillydrone Avenue, Aberdeen AB24 2TZ, UK

*Author for correspondence (e-mail: voigt@izw-berlin.de)

Accepted 7 May 2008

SUMMARY

The stable carbon isotope ratio of exhaled CO_2 ($\delta^{13}C_{breath}$) reflects the isotopic signature of the combusted substrate and is, therefore, suitable for the non-invasive collection of dietary information from free-ranging animals. However, $\delta^{13}C_{breath}$ is sensitive to changes in ingested food items and the mixed combustion of exogenous and endogenous substrates. Therefore, experiments under controlled conditions are pivotal for the correct interpretation of $\delta^{13}C_{breath}$ of free-ranging animals. We measured $\delta^{13}C_{breath}$ in fasted and recently fed insectivorous *Myotis myotis* (Chiroptera) to assess the residence time of carbon isotopes in the pool of metabolized substrate, and whether $\delta^{13}C_{breath}$ in satiated individuals levels off at values similar to the dietary isotope signature ($\delta^{13}C_{diet}$) in insect-feeding mammals. Mean $\delta^{13}C_{breath}$ of fasted individuals was depleted by –5.8‰ (*N*=6) in relation to $\delta^{13}C_{diet}$. After feeding on insects, bats exchanged 50% of carbon atoms in the pool of metabolized substrates within 21.6±10.5 min, which was slower than bats ingesting simple carbohydrates. After 2 h, $\delta^{13}C_{breath}$ of satiated bats levelled off at –2.6‰ below $\delta^{13}C_{diet}$, suggesting that bats combusted both exogenous and endogenous substrate at this time. A literature survey revealed that small birds and mammals metabolize complex macronutrients at slower rates than simple macronutrients. On average, $\delta^{13}C_{breath}$ of fasting birds and mammals was depleted in ^{13}C by –3.2±2.0‰ in relation to $\delta^{13}C_{diet}$. $\delta^{13}C_{breath}$ of satiated animals differed by –0.6±2.3‰ from $\delta^{13}C_{diet}$ when endogenous substrates were not in isotopic equilibrium with exogenous substrates and by +0.5±1.8‰ (*N*=6 species) after endogenous substrates were in isotopic equilibrium with exogenous substrates.

Key words: bats, dietary preferences, exogenous substrate, fat, metabolism, stable isotopes.

INTRODUCTION

During the past decades, ecologists have increasingly used stable isotopes to study trophic interactions and dietary preferences in animal food webs (reviewed in Fry, 2006). Animals assimilate stable isotopes from the diet into their tissue or release them in their excreta and exhaled breath (DeNiro and Epstein, 1978; DeNiro and Epstein, 1981). Usually, excreta are depleted in ^{13}C in relation to the stable carbon isotope signature of the diet, whereas animal tissues are enriched in ^{13}C in relation to the diet by enzymatic action called isotopic fractionation (DeNiro and Epstein, 1978; DeNiro and Epstein, 1981). Isotopes are assimilated into animal tissue according to organ-specific tissue turnover rates (e.g. Tieszen et al., 1981). Thus, stable isotope signatures in organs with different turnover rates shed light on the diet consumed by the animal during the corresponding time periods. The stable carbon isotope composition ($\delta^{13}C$) of bone is considered to integrate the stable isotope ratio of the food consumed during an animal's lifetime (e.g. Tieszen and Fagre, 1993). The stable carbon isotope ratio of other tissues, such as muscle, fat and liver, provides information on shorter time periods (Tieszen et al., 1981). The isotopic incorporation rates into these tissues have been found to be variable (Dalerum and Angerbörn, 2005) and possibly linked to the mass of the mammal (Carleton and Martínez del Rio, 2005). The stable carbon isotope signature of exhaled breath ($\delta^{13}C_{breath}$) is considered to be the fastest recorder of dietary information, because $\delta^{13}C_{breath}$ should match the isotopic signature of the ingested and combusted substrate (Perkins and Speakman, 2001; Hatch et al., 2002a; Hatch et al., 2002b).

The earliest experiments conducted under controlled conditions supported the idea that $\delta^{13}C_{breath}$ closely matches the isotopic composition of the recently ingested food ($\delta^{13}C_{diet}$) (DeNiro and Epstein, 1981; Klein et al., 1988; Tieszen and Fagre, 1993). Since $\delta^{13}C_{breath}$ seems to provide a snapshot of the stable isotope signature of the substrate currently metabolized, it is increasingly used by experimental physiologists and behavioural ecologists to study metabolic substrate use in captive (e.g. Carleton et al., 2006) and free-ranging animals (e.g. Podlesak et al., 2005). Diet-switching experiments during which the isotopic composition of the diet is drastically changed at the onset of the experiment – from e.g. plant products of C3 plant origin to those of C4/CAM plant origin – have provided important insights into substrate combustion in birds (e.g. Hatch et al., 2002a; Hatch et al., 2002b; Carleton et al., 2006) and mammals (e.g. Jeuckendrup and Jentjens, 2000; Voigt and Speakman, 2007). During diet-switch experiments, breath samples are collected at regular time intervals after animals have started to consume a meal differing in stable carbon isotope composition from their previous diet. Such experiments have revealed that simple sugars are routed to combustion within less than 10 min in small birds and mammals (Voigt and Speakman, 2007; Welch et al., 2006; Welch et al., 2008; Voigt et al., 2008a) and in approximately 30 min in

exercising humans (Jeuckendrup and Jentjens, 2000). Complex macronutrients such as cellulose, starch and proteins are probably more difficult to digest than simple sugars, since the number of enzymatic processes is likely to increase with increasing complexity of the catabolized macronutrient. Therefore, animals ingesting complex macronutrients may incorporate exogenous substrate at slower rates than animals ingesting simple sugars.

In addition to differences in the time lag at which macronutrient combustion is reflected in $\delta^{13}C_{breath}$, $\delta^{13}C_{breath}$ may be affected by other factors such as isotopic fractionation during enzymatic or physical processes, or by the combined use of exogenous and endogenous substrates. Endogenous substrates are usually depleted in ^{13}C by –0.6 to –8.4‰ in relation to the diet at the time of lipogenesis (e.g. DeNiro and Epstein, 1977; Podlesak et al., 2006). Accordingly, $\delta^{13}C_{breath}$ of fasting animals is usually depleted in ^{13}C in relation to the previous diet (e.g. Hatch et al., 2002a; Hatch et al., 2002b). In many animals, especially free-ranging animals, it may not be evident whether individuals combust exclusively endogenous substrates or a mixture of exogenous and endogenous substrates. In this case, the difference between $\delta^{13}C_{breath}$ and $\delta^{13}C_{diet}$, henceforth called $\Delta_{diet–breath}$, may be affected by the isotopic composition of both substrates and the ratio at which they are used. Following this line of argument, if endogenous substrates are not in isotopic equilibration with the most recent diet and if animals metabolize both types of substrate, $\Delta_{diet–breath}$ may largely deviate from the most recent $\delta^{13}C_{diet}$ (e.g. Podlesak et al., 2005; Carleton et al., 2006). Continuous ingestion of new food items with a $\delta^{13}C$ signature differing from that of the previous diet will eventually cause an exchange of isotopes in endogenous reserves over time. Thus, $\Delta_{diet–breath}$ should decrease with increasing equilibration of fat and glycogen to the new $\delta^{13}C_{diet}$ (Carleton et al., 2006).

Considering all these factors that may have a large and short-term impact on $\delta^{13}C_{breath}$ and consequently $\Delta_{diet–breath}$, the aim of our study was (1) to generate baseline data of stable isotope turnover and $\Delta_{diet–breath}$ for a protein-combusting animal and (2) to survey the literature for general patterns to improve our ability to interpret stable isotope breath data of free-ranging animals.

First, using small insectivorous greater mouse-eared bats (*Myotis myotis*, Vespertilionidae), we quantified $\Delta_{diet–breath}$ in fasting individuals, the rate at which exogenous substrates are incorporated into the pool of metabolized substrates after ingestion of a protein-rich diet and the plateau $\delta^{13}C_{breath}$ at which the animals' breath levelled off after equilibration to the new diet. We predicted that (1) the rate at which *M. myotis* would make use of dietary proteins for metabolism would be slower than in sugar-combusting bats of similar size, (2) $\delta^{13}C_{breath}$ should be depleted in ^{13}C in relation to $\delta^{13}C_{diet}$ in fasting animals, and (3) $\delta^{13}C_{breath}$ should level off close to $\delta^{13}C_{diet}$ in satiated animals.

Second, we reviewed the current knowledge on species-specific $\Delta_{diet–breath}$ values and fractional rates at which exogenous substrates are incorporated into the pool of metabolized substrates for birds and mammals. We predicted that (1) animals ingesting complex macronutrients, such as proteins and complex carbohydrates, would have a slower fractional incorporation rate of exogenous substrate into the pool of metabolized substrate than animals feeding on simple carbohydrates such as hexose or sucrose, (2) fasting birds and mammals would have, in general, negative $\Delta_{diet–breath}$ values, since endogenous substrates are usually depleted in ^{13}C in relation to the diet, and (3) $\Delta_{diet–breath}$ should decrease in satiated animals with increasing equilibration of endogenous substrate to the isotopic signature of the diet.

MATERIALS AND METHODS

Study animals and isotopic signature of experimental food types

We studied metabolic substrate use in six male adult *Myotis myotis* Borkhausen 1797 in a captive population housed in facilities at the Max-Planck-Institute for Ornithology in Seewiesen. *M. myotis* is an insectivorous bat that forages on carabid beetles in temperate zone forests (Arlettaz et al., 1997). Our study animals were kept under an inverted dark–light cycle. The dark phase, i.e. the activity period, started at 09:00 h and lasted until 17:00 h. As exclusive bat food for the duration of the experiment, we raised mealworms (larval stages of *Tenebrio molitor*) on corn from either Hungary or Ecuador for 7 weeks. Ten days before the breath sampling, bats were fed mealworms enriched in ^{13}C with corn from Hungary. Previous experiments in other bats demonstrated that this time period is sufficient to equilibrate the endogenous reserves of bats isotopically to a new diet (Voigt and Speakman, 2007). During the experiment, bats were fed with mealworms that were enriched in ^{13}C with corn from Ecuador. We collected several mealworms from each diet for later stable isotope analyses. Dead mealworms were dried to constant mass at 40°C in a drying oven and then a subsample from the tissue of inner organs was taken for stable carbon isotope analyses. The mealworms raised for 7 weeks on Hungarian corn had a $\delta^{13}C$ of –24.1±0.8‰ (diet 1), while those raised on the Ecuadorian corn had a $\delta^{13}C$ of –22.0±0.3‰ (diet 2), indicating that the isotopic signature of the mealworms was slightly more enriched in ^{13}C than before.

Incorporation rate of exogenous substrate into the pool of metabolized substrates

We assessed the rate at which exogenous substrate was incorporated into the pool of metabolized substrates in a diet-switch experiment, i.e. we fed fasted bats that had been maintained over 6 or 7 days *ad libitum* on diet 1 with diet 2. Prior to the diet-switch experiment, bats fasted for at least 13 h, which is fully congruent with the bats' natural diurnal pattern of food intake. All experiments were performed during the activity period of the bats.

Before offering mealworms, we collected an initial breath sample from each bat. Following the ingestion of the first mealworm we collected breath samples after 10, 20, 40, 60, 80, 100 and 120 min (see below for the technique of breath collection). Bats were weighed to the nearest 0.1 g before and after the experiment using a hand-held balance (Pesola, Baar, Switzerland). We lost body mass data from two individuals for the time after the experiment and, therefore, can only provide body mass change data for four individuals.

Breath collection

For breath sampling, bats were transferred singly into cotton bags (17 cm×25 cm) that were individually put into a larger plastic bag (volume 0.5 ml; Ziplock™, Racine, WI, USA). Ambient air was washed of CO_2 using NaOH and flushed through the bag *via* a plastic tube (diameter 3 mm) at a minimal flow through rate of 1.4 l min^{-1}. The outlet of the plastic bag consisted of a small slit of 4 cm (width 0.2 cm). A small tube was positioned with one end close to the bat's head inside the bag (diameter 1 mm, length 4 cm). We fused a needle hermetically to the other end of the tube outside the bag. For CO_2 accumulation, we sealed the plastic bag for 1 min. *M. myotis* have a resting metabolic rate of approximately 25 ml O_2 h^{-1} (Hanu, 1959). Therefore, we expected CO_2 to accumulate to approximately 0.5% during this time span. We then sucked air from the bag including the bat's breath into a vacutainer (Labco, Buckinghamshire, UK) by penetrating the Teflon membrane of the vacutainer with the

needle. After each breath collection the plastic bag was unsealed again and CO_2-free air was flushed through the bag. Breath collection was repeated after 10, 20, 40, 60, 80, 100 and 120 min following the first feeding event, since we expected an exchange of stable carbon isotopes in exhaled CO_2 during this time period (Voigt & Speakman, 2007; Voigt et al., 2008a). Bats were fed repeatedly after 20, 40 and 60 min following the first feeding event to ensure that the bat's breath was equilibrated isotopically to the new diet. We define isotopic equilibration of an animal's tissue or breath as the status in which the isotopic composition of animal tissue or breath does not change any more with continuous consumption of the same food items.

Stable carbon isotope analysis

Breath samples were measured with an Isochrom-μG isotope ratio mass spectrometer (Micromass, UK) (Perkins and Speakman, 2001; Voigt and Speakman, 2007). Samples were automatically flushed from the vacutainers in a stream of chemically pure helium, after which a gas chromatograph separated the CO_2 gas from the other gases before admitting it into the mass spectrometer in a continuous flow. Breath samples together with internal standards that had previously been characterized relative to an international ^{13}C standard (IAEA-CO-1) were analysed in duplicate. All $^{13}C/^{12}C$ ratios were expressed relative to the international standard in δ notation (‰) using the following equation:

$$\delta^{13}C = \{[(^{13}C/^{12}C)_{sample}/(^{13}C/^{12}C)_{standard}] - 1\} \times 10^3 , \quad (1)$$

with $^{13}C/^{12}C$ representing the isotope ratio in either the breath sample or the standard. Precision was better than ±0.01‰ (1 s.d.). All samples were analysed using a blind experimental protocol.

Subsamples from the bats' diet (inner organs of the mealworms, excluding the cuticula) were dried at 40°C in a drying oven to constant mass, weighed on a Sartorius microbalance (Satorius AG, Göttingen, Germany) and loaded into tin capsules. All samples were combusted and analysed with a Flash elemental analyser and a Conflo II, coupled to a Delta-Advantage isotope ratio mass spectrometer (FisherThermo, Bremen, Germany) at the Stable Isotope Laboratory of the Leibniz-Institute for Zoo and Wildlife Research, Berlin (Germany). Samples were analysed in combination with internal standards that had previously been characterized relative to an international ^{13}C standard (NBS22). All $^{13}C/^{12}C$ data were expressed relative to the international standard in the δ notation (‰) using Eqn 1. Precision was better than ±0.03‰ (1 s.d.).

Regression model

We expected changes in isotopic composition to follow an exponential model (e.g. Tieszen et al., 1983; Voigt and Speakman, 2007) and used a one-compartment model instead of a multi-compartment model because we were not able to estimate five or more regression parameters with eight data points [for a mathematical approach for the use of multi-compartment models, see Martínez del Rio and Anderson-Sprecher (Martínez del Rio and Anderson-Sprecher, 2008)]. Therefore, we calculated equations of the following type for each of the *M. myotis* according to Carleton and Martínez del Rio (Carleton and Martínez del Rio, 2005):

$$\delta^{13}C_{breath}(t) = \delta^{13}C_{breath}(\infty) + [\delta^{13}C_{breath}(0) - \delta^{13}C_{breath}(\infty)]\, e^{-t/k} , \quad (2)$$

where $\delta^{13}C_{breath}(t)$ is the stable carbon isotope ratio of exhaled CO_2 at time t, $\delta^{13}C_{breath}(\infty)$ is the asymptotic stable carbon isotope ratio of exhaled CO_2 when animals are equilibrated to the stable carbon isotope signature of their diet, $\delta^{13}C_{breath}(0)$ is the stable isotope ratio of exhaled CO_2 at time 0 of the experiment, and k is the residence time (min) of isotopes in the pool of metabolized substrates. Estimation of k was performed on an iterative basis using Systat (Systat Software Inc., version 11.00.01, San José, CA, USA). We used the mean regression coefficients of all individual regression curves to derive a species-specific regression equation for the residence time of carbon atoms in the pool of metabolized substrate in *M. myotis*. To test for differences in mean $\delta^{13}C_{breath}(\infty)$ and the stable carbon isotope ratio of the diet, we performed Wilcoxon rank-sum tests.

For comparative reasons, we calculated the time at which 50% of carbon isotopes were exchanged in the animal's breath (t_{50}) according to the following equation: $t_{50}=-\ln(0.5)k$, with ln representing the natural logarithm and 0.5 the exchange of 50% of isotopes. All values are given as means ± 1 s.d. and all statistical tests were performed two tailed unless otherwise stated.

Literature survey

We surveyed the literature for experimental studies that used $\delta^{13}C_{breath}$ as a predictor for $\delta^{13}C_{diet}$ in birds and mammals. For each study, we obtained the following six parameters: (a) taxonomic group, (b) body mass in grams, (c) t_{50} (min), or the fractional incorporation rate at which 50% of carbon atoms in exhaled CO_2 have been exchanged with carbon atoms from the exogenous substrate, (d) $\Delta_{diet-breath}$ for animals having fasted over a prolonged time period, (e) $\Delta_{diet-breath}$ for animals having ingested a diet that was more enriched in ^{13}C in relation to the previous diet (exogenous substrate with a C4 signature and endogenous substrate with a C3 signature and, thus, endogenous substrate not in isotopic equilibrium with the new diet), and (f) $\Delta_{diet-breath}$ for animals having ingested a diet that was isotopically identical to the previous diet (endogenous substrate in isotopic equilibrium with the new diet). In cases where we obtained two or more values for one of these parameters, we calculated an arithmetic mean for these data to yield a single species-specific data point. We calculated a Wilcoxon matched-pairs signed-rank test for comparison of $\Delta_{diet-breath}$ between scenarios d and e, and d and f, and a Mann-Whitney *U*-test for a comparison of $\Delta_{diet-breath}$ between scenarios e and f. All three tests were performed one tailed, as we expected that the exclusive or additional combustion of endogenous substrates would cause a depletion of ^{13}C in exhaled breath. We calculated a Mann–Whitney *U*-test for comparison of the t_{50} values in animals digesting complex substrates, such as starch- or protein-rich diets, and animals digesting simple substrates, such as hexose or sucrose. This analysis was only performed for mammals and birds weighing less than 1 kg, as we expected a large impact of passage rate and endosymbiontic fermentation on t_{50} values in large mammals (e.g. in ruminants).

RESULTS

Isotopic change of $\delta^{13}C_{breath}$ in insect-feeding *Myotis myotis*

The body mass of fasted *M. myotis* averaged 23.1±1.9 g 1 h after the onset of the dark cycle. The mean $\delta^{13}C_{breath}$ of fasted *M. myotis* equalled –29.9±0.7‰, which was –5.8‰ lower than the $\delta^{13}C$ of the diet (–24.1‰) that the bats fed on during the 6–7 days preceding the day of breath collection; this difference was significant (Wilcoxon signed-rank test: $T+=0$, $T-=-21$, $P=0.0156$). After being fed continuously with mealworms ($\delta^{13}C=-22.0$‰), $\delta^{13}C_{breath}$ became enriched in ^{13}C (Fig. 1). The body mass of bats increased until the end of the feeding experiment by on average 4.4±1.6 g due to the ingestion of mealworms. The regression model calculated for the fractional incorporation rate of exogenous substrate into the pool

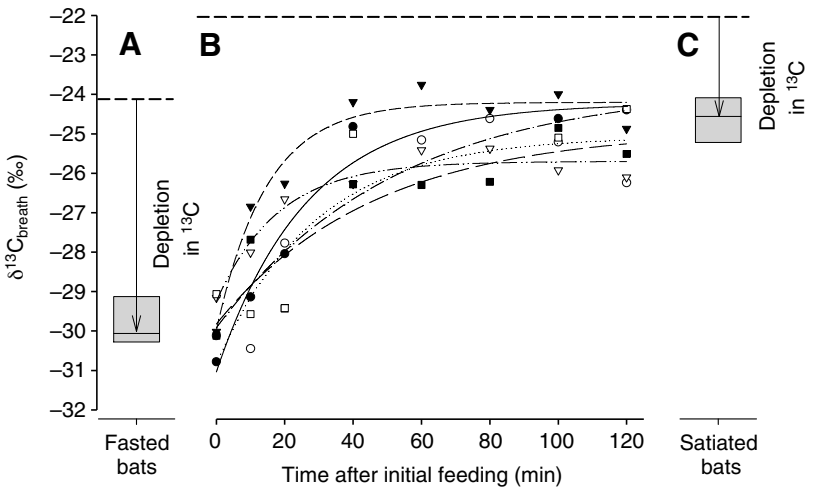

Fig. 1. Stable carbon isotope ratio of exhaled breath ($\delta^{13}C_{breath}$) in six *Myotis myotis* before (A), during (B) and after (C) they were fed mealworms. Before the experiment, bats had been maintained on a diet of mealworms with a stable carbon isotope signature of –24.1‰ (broken line in A). During the experiment, bats were fed mealworms with an isotopic signature of –22‰ (broken line in B and C). Regression curves in B were calculated following a single-pool exponential model for each individual (each individual is represented by individual symbols and regression curves). Values in A and C are presented as box plots with the borders of the boxes depicting the 25 and 75 percentiles and the solid line within the box depicting the median. Exhaled breath of fasted bats was depleted by approximately –5.8‰ in ^{13}C in relation to the dietary isotope signature ($\delta^{13}C_{diet}$, A), whereas exhaled breath was only depleted by approximately –2.6‰ in ^{13}C in relation to $\delta^{13}C_{diet}$ in satiated bats (estimated individual plateaus of the exponential regression models; C).

of metabolized substrate reads as follows: $\delta^{13}C_{breath}(t)= -24.6(\pm0.7)-5.5(\pm1.1)e^{-t/31.2(\pm15.2)}$. The mean time required to exchange 50% of the carbon atoms in the exhaled CO_2 with carbon atoms of the recently ingested protein source was 21.6±10.5 min (Table 1). The $\delta^{13}C_{breath}$ levelled off at –24.6±0.7‰ after 120 min, which was –2.6‰ lower than the $\delta^{13}C$ of the new diet (Wilcoxon signed-rank test: $T+=0$, $T-=-21$, $P=0.0313$).

t_{50} and $\Delta_{diet-breath}$ in birds and mammals

The fractional rates of incorporation of exogenous substrates into the pool of metabolized substrates ranged from a few minutes in nectar-feeding small birds and bats to almost 3 days in hay-ingesting alpacas (see Table 2). In species below 1 kg body mass, animals had higher t_{50} values when digesting complex substrates such as a starch- or protein-rich diet than when digesting simple substrates such as hexose or sucrose (Mann–Whitney *U*-test: $U=0.0$, $U'=25$, $N_1=5$, $N_2=5$, $P=0.0079$). In fasting birds and mammals, mean $\delta^{13}C_{breath}$ was depleted in relation to $\delta^{13}C_{diet}$ by –3.2±2.0‰ ($N=4$ bird and $N=5$ mammal species; Table 2, Fig. 2). In animals that had recently fed on a diet with a C4 signature but still carried endogenous reserves with a C3 signature, $\delta^{13}C_{breath}$ was depleted in ^{13}C by –0.6±2.3‰ in relation to the $\delta^{13}C$ of the new diet ($N=5$ bird and $N=7$ mammal species). After continuously feeding on the new diet, the endogenous reserves (fat and glycogen) became isotopically equilibrated to the new diet. $\delta^{13}C_{breath}$ was, on average, enriched in ^{13}C by +0.5±1.8‰ in relation to the diet after isotopic equilibration of endogenous substrate with the diet ($N=6$ species; Fig. 2). Depletion of ^{13}C in exhaled breath in relation to $\delta^{13}C_{diet}$ was stronger in fasting animals than in animals that had recently fed on an isotopically contrasting diet (Wilcoxon matched-pairs signed-rank test: $T+=7$, $T-=-38$, $N=9$ pairs, $P=0.0371$; scenario d–e) and also more pronounced than in animals carrying endogenous substrates already isotopically equilibrated to the diet (Mann–Whitney *U*-test: $U=2.0$, $U'=38$, $N_1=9$, $N_2=5$, $P=0.0062$; Fig. 2; scenario d–f). On average, $\Delta_{diet-breath}$ was not significantly different between satiated animals with endogenous substrates equilibrated to the new diet and satiated animals with endogenous substrates not equilibrated to the new diet (Mann–Whitney *U*-test: $U=19$, $U'=36$, $N_1=12$, $N_2=5$, $P=0.377$; Fig. 2; scenario e–f).

Table 1. Individual parameters for the single-pool exponential regression model calculated for the fractional incorporation rate of ingested proteins into the pool of metabolized substrates in six *Myotis myotis*

Individual	$\delta^{13}C_{breath}(\infty)$ (‰)	$\delta^{13}C_{breath}(0)-\delta^{13}C_{breath}(\infty)$ (‰)	k (min)	r^2_{corr}	t_{50} (min)
1	–24.2	–6.8	26.4	0.963	18.3
2	–25.1	–5.8	29.4	0.949	20.4
3	–24.2	–5.8	14.7	0.973	10.2
4	–25.7	–3.6	17.9	0.975	12.4
5	–24.9	–4.9	45.7	0.822	31.7
6	–23.8	–6.2	53.0	0.930	36.7
Mean ± 1 s.d.	–24.6±0.7	–5.5±1.1	31.2±15.2		21.6±10.5

$\delta^{13}C_{breath}(\infty)$, asymptotic stable carbon isotope ratio of exhaled CO_2 when animals are equilibrated to the stable carbon isotope signature of their diet; $\delta^{13}C_{breath}(0)$, stable isotope ratio of exhaled CO_2 at time 0; k, residence time of isotopes in the pool of metabolized substrates; and t_{50}, time at which 50% of carbon isotopes were exchanged in the animal's breath.

DISCUSSION

Fractional incorporation rates in mammals and birds

We fed fasting vespertilionid bats (*Myotis myotis*) mealworms and monitored changes in the bats' $\delta^{13}C_{breath}$ over a 2 h period. The $\delta^{13}C_{breath}$ of the bats converged quickly on the stable carbon isotope signature of the newly ingested food items. *M. myotis* required twice as much time to exchange 50% of carbon atoms in exhaled CO_2 with those of the last meal as similar-sized *Carollia perspicillata* (family Phyllostomidae) feeding on simple sugars (Voigt et al., 2008a), but only 70% of the time similar-sized vampire bats need to metabolize recently ingested blood (Voigt et al., 2008). Mice (*Mus musculus*) feeding on corn, which includes mainly complex carbohydrates, i.e. starch, had almost identical t_{50} values to *M. myotis* (Perkins and Speakman, 2001). The lower t_{50} values of sugar-ingesting bats may be caused by the faster enzymatic processing of small molecules, such as hexose or sucrose, than more complex molecules, such as starch and proteins. Our literature survey supports the notion that animals require more time to digest and combust complex macronutrients. However, t_{50} values may be affected not only by the type of combusted macronutrient but also by the animal's size and phylogenetic background (Carleton and Martínez del Rio, 2005). Currently, we lack sufficient data to shed light on the effects of phylogenetic inertia on t_{50} values. Data for t_{50} listed in Table 2 suggest that large animals

Table 2. Fractional incorporation rates of exogenous substrate into the pool of metabolized substrate and isotopic differences between diet and exhaled CO_2 ($\Delta_{diet-breath}$) in birds and mammals based on a literature survey

Species	Taxon	Body mass (g)	t_{50} (min)	Δ_a (‰)	Δ_b (‰)	Δ_c (‰)	Macronutrient (food source)	Source
Glossophaga soricina	Ch.	10	12	–0.5	–2.9	n.a.	SCH (nectar*)	(Voigt and Speakman, 2007)
			9‡	–0.1	n.a.	n.a.	SCH (nectar*)	(Welch et al., 2008)
Carollia perspicillata	Ch.	20	11.5	–3.0	–2.0	n.a.	SCH (nectar*)	(Voigt et al., 2008a)
Myotis myotis	Ch.	23	21.5	–5.8	–2.6	n.a.	P (insects)	This study
Desmodus rotundus	Ch.	30	29.5	–4.6	+2.2	n.a.	P (blood)	(Voigt et al., 2008b)
Mus musculus	Ro.	37	20.4†	–5.7	–3.7	n.a.	CCH (corn)	(Perkins and Speakman, 2001)
Microtus ochrogaster	Ro.	50	n.a.	n.a.	n.a.	+0.3	CCH + P (pellets)	(Passey et al., 2005)
Oryctolagus cuniculus	La.	1800	n.a.	n.a.	n.a.	+1.0	CCH + P (pellets)	(Passey et al., 2005)
Sus scrofa	Ar.	70 000	n.a.	n.a.	n.a.	+1.8	CCH + P (pellets)	(Passey et al., 2005)
Homo sapiens	Pr.	74 000	30.4†,‡	n.a.	n.a.	n.a.	SCH (nectar*)	(Jeukendrup and Jentjens, 2000)
Lama pacos	Ar.	135 000	4032†	n.a.	+2.4	n.a.	CCH (hay)	(Sponheimer et al., 2006)
Equus ferus	Pe.	600 000	288§	n.a.	+2.0	n.a.	CCH (hay)	(Ayliffe et al., 2004)
Bos taurus	Ar.	650 000	n.a.	n.a.	n.a.	+2.9; +2.7	CCH (hay)	(Passey et al., 2005; Metges et al., 1990)
Selasphorous platycercus	Ap.	3	10.9†	–5.5	–4.9	n.a.	SCH (nectar*)	(Welch et al., 2006)
			n.a.	–2.9	–1.7	–1.3	SCH (nectar*)	(Carleton et al., 2006)
Selasphorous rufus	Ap.	3.4	8.8†	–0.3	–0.7	n.a.	SCH (nectar*)	(Welch et al., 2008)
Calypte annae	Ap.	4.8	5.9†	–1.9	–0.9	n.a.	SCH (nectar*)	(Welch et al., 2008)
Dendroica coronata	Pa.	12	264	n.a.	+0.9	n.a.	SCH+P+F (agar based)	(Podlesak et al., 2005)
Columbia livia	Co.	330	210	–3.0	+1.3	n.a.	CCH (corn)	(Hatch et al., 2002b)

Species were sorted according to increasing body mass, first for mammals then for birds. Values for $\Delta_{diet-breath}$ are listed for three different scenarios: (a) animals fasted (Δ_a), (b) animals fed but endogenous substrate not equilibrated to new diet (Δ_b), and (c) animals fed and endogenous substrate equilibrated to diet (Δ_c).

SCH, simple carbohydrates; CCH, complex carbohydrates; P, protein; F, fat; n.a., data not available.

*We define nectar as an artificial sugary water solution. †Calculated using data in source papers. ‡Exercise metabolism. §Calculated after a three-pool model (fastest pool).

Mammalia: Ar., Artiodactycla; Ch., Chiroptera (Mammalia); Pe., Perissodactyla; Pr., Primates; Ro., Rodentia. Aves; Ap., Apodiformes; Co., Columbiformes; Pa., Passeriformes.

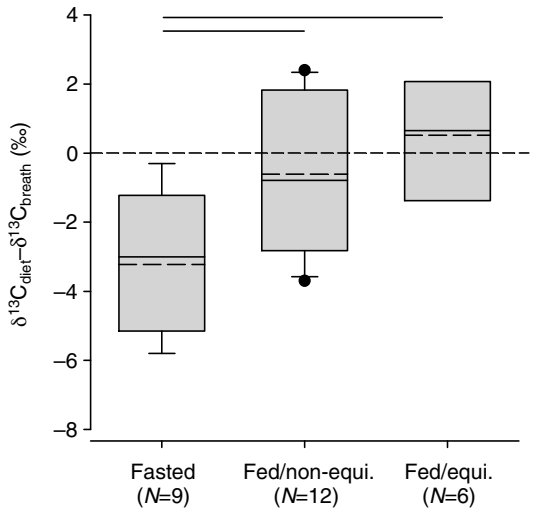

Fig. 2. Isotopic difference between diet and exhaled CO_2 in various mammals and birds, when fasting, when fed but endogenous substrate not isotopically equilibrated to the new diet (Fed/non-equi.), and when fed and endogenous substrate isotopically equilibrated to the new diet (Fed/equi.). The broken line across the box plots indicates a perfect match of $\delta^{13}C_{breath}$ with $\delta^{13}C_{diet}$. Species-specific data and sources are listed in Table 2. The borders of the box plots represent the 25 and 75 percentiles, T-marks are the 5 and 95 percentiles, and filled circles are outliers. Solid lines within the boxes depict the median and broken lines within the boxes the mean value. Solid lines above the box plots depict significant differences among groups.

have slower incorporation rates (t_{50} values) than small animals. Despite being large, alpacas and horses make use of complex carbohydrates such as cellulose by endosymbiontic fermentation. This mode of digestion may additionally slow down the speed at which exogenous substrates are available to fuel metabolism. Our survey also highlighted the fact that t_{50} values were lower when the focus organisms were exercising [see reports on humans (Jeukendrup and Jentjens, 2000) and bats (Welch et al., 2008)]. However, the effect of exercise may become negligible in small animals ingesting simple sugars, as t_{50} values were almost identical for resting and flying nectar-feeding bats feeding on a sugar water solution (Voigt and Speakman, 2007; Welch et al., 2008).

Isotopic differences between diet and exhaled CO_2

The $\delta^{13}C_{breath}$ of *M. myotis* fasting for 13 h was depleted in ^{13}C by almost –6‰ in relation to the $\delta^{13}C_{diet}$ of the animal's previous diet. In general, $\Delta_{diet-breath}$ averaged –3.2±2.0‰ for nine bird and mammal species that had fasted over an extended period of time (Table 2, Fig. 2). Thus, a depletion of ^{13}C in relation to the isotopic signature of the diet ($\Delta_{diet-breath}$) seems to be a common phenomenon in fasting animals and is probably caused by the strong fractionation of stable carbon isotopes during lipogenesis (DeNiro and Epstein, 1977).

After we fed fasting *M. myotis* with mealworms (diet 2) with a stable carbon isotope signature that was enriched by +2‰ in relation to the bats' previous food (diet 1), the bats' $\delta^{13}C_{breath}$ converged to a new plateau, which was depleted by –2.6‰ in relation to the $\delta^{13}C$ of the new diet. By contrast, $\delta^{13}C_{breath}$ of blood-feeding vampire bats (*Desmodus rotundus*) levelled off +2‰ above the $\delta^{13}C$ of their recently ingested blood meal (Voigt et al., 2008b). Given that both bat species process and metabolize protein, and assuming similar

fractionation factors during protein combustion, *M. myotis* probably used more ^{13}C-depleted endogenous substrate to fuel its metabolism than *D. rotundus*.

Many previous studies have looked at the relative level of the animals' $\delta^{13}C_{breath}$ plateau values after a diet switch (see Table 2). During most diet-switch experiments animals used a combination of exogenous and endogenous substrates to fuel their metabolism and in most cases experimental animals were labelled with carbon atoms from a C3 food web (low $\delta^{13}C$) at the beginning of the experiment. Since fat and glycogen stores of these experimental animals were built from carbon atoms of a C3 food source, $\Delta_{diet-breath}$ may deviate from zero throughout the experiment, because the exogenous substrate combustion in relation to the endogenous substrate combustion may vary over time (e.g. Carleton et al., 2006). On average, $\Delta_{diet-breath}$ equalled –0.6±2.3‰ in 12 bird and mammal species that had recently ingested food with a C4 signature, but still carried endogenous reserves with a C3 signature (Table 2, Fig. 2). After isotopic equilibration of endogenous substrate with the new diet, average $\Delta_{diet-breath}$ equalled +0.5±1.8‰ (N=6 species; Fig. 2). Thus, $\Delta_{diet-breath}$ decreased with increasing equilibration of endogenous substrates with the stable carbon isotope signature of the new diet.

Our survey highlights the fact that $\delta^{13}C_{breath}$ may be affected not only by the stable carbon isotope signature of the recently ingested food but also by the time elapsed since an isotopic shift in the diet, the turnover rate of endogenous substrates and the ratio at which endogenous and exogenous substrates are used for metabolism, e.g. when active or resting. Thus, animals that have recently changed to food items with an isotopic composition different from their previous diet may exhibit the largest range in $\Delta_{diet-breath}$. Interpretation of $\delta^{13}C_{breath}$ may prove difficult if no alternative measure of substrate combustion is available, e.g. the respiratory quotient of exhaled breath or metabolic products in blood plasma. We, therefore, advise caution in the interpretation of $\delta^{13}C_{breath}$ from free-ranging animals without further validation studies under controlled experimental conditions, preferably in the same animal species as investigated in the field. The sensitivity of $\delta^{13}C_{breath}$ to various factors such as fractionation, mixed combustion of exogenous and endogenous substrates and others may provide a powerful tool for physiologists and behavioural ecologists, once the underlying mechanisms are clearly understood.

Peter Thompson and Dr Paula Redman analysed the samples at the Aberdeen Centre for Energy Regulation and Obesity, and Karin Sörgel at the Stable Isotope Laboratory of the IZW. The experiments complied with the current laws of Germany; animals were caught and kept under licence of the responsible regulatory authorities; Regierungspräsidium Freiburg, licence no. 55-8852.44/1095, Veterinäramt Starnberg). We thank Carlos Martínez del Rio, Scott Carleton and Detlev H. Kelm for commenting on an earlier version of this manuscript.

REFERENCES

Arlettaz, R., Perrin, N. and Hausser, J. (1997). Trophic ressources partitioning and competition between the two sibling bat species *J. Anim. Ecol.* **66**, 897-911.

Ayliffe, L. K., Cerling, T. E., Robinson, T., West, A., Sponheimer, M., Passey, B. H., Hammer, J., Roeder, B., Dearing, M. D. and Ehleringer, J. R. (2004). Turnover of carbon isotopes in tail hair and breath CO_2 of horses fed an isotopically varied diet. *Oecologia* **139**, 11-22.

Carleton, S. A. and Martínez del Rio, C. (2005). The effect of cold-induced increased metabolic rate on the rate of ^{13}C and ^{15}N incorporation in house sparrows (*Passer domesticus*). *Oecologia* **144**, 226-232.

Carleton, S. A., Bakken, B. H. and Martínez del Rio, C. (2006). Metabolic substrate use and the turnover of endogenous energy reserves in broad-tailed hummingbirds (*Selasphorus platycercus*). *J. Exp. Biol.* **209**, 2622-2627.

Dalerum, F. and Angerbjörn, A. (2005). Resolving temporal variation in vertebrate diets using naturally occurring stable isotopes. *Oecologia* **144**, 647-658.

DeNiro, M. J. and Epstein, S. (1977). Mechanism of carbon isotope fractionation associated with lipid synthesis. *Science* **197**, 261-263.

DeNiro, M. J. and Epstein, S. (1978). Influence of diet on the distribution of carbon isotopes in animals. *Geochim. Cosmochim. Acta* **42**, 495-506.

DeNiro, M. J. and Epstein, S. (1981). Influence of diet on the distribution of nitrogen isotopes in animals. *Geochim. Cosmochim. Acta* **45**, 341-351.

Fry, B. (2006). *Stable Isotope Ecology*. Berlin: Springer.

Hanu, K. (1959). Body temperatures and metabolism in bats at different environmental temperatures. *Physiol. Bohem.* **8**, 250-259.

Hatch, K. A., Pinshow, B. and Speakman, J. R. (2002a). Carbon isotope ratios in exhaled CO_2 can be used to determine not just present, but also past diets in birds. *J. Comp. Physiol. B* **172**, 263-268.

Hatch, K. A., Pinshow, B. and Speakman, J. R. (2002b). The analysis of $^{13}C/^{12}C$ ratios in exhaled CO_2: its advantages and potential application to field research to infer diet, changes in diet over time, and substrate metabolism in birds. *Integr. Comp. Biol.* **42**, 21-33.

Jeuckendrup, A. E. and Jentjens, R. (2000). Oxidation of carbohydrate feedings during prolonged exercise – current thoughts, guidelines and directions for future research. *Sports Med.* **69**, 407-424.

Klein, H.-J., Schulze, E., Deegen, E. and Gies, W. (1988). Metabolism of naturally occuring [^{13}C]glucose given orally to horses. *Am. J. Vet. Res.* **8**, 1259-1262.

Martínez del Rio, C. and Anderson-Sprecher, R. (2008). Beyond the reaction progress variable: the meaning and significance of isotopic incorporation data. *Oecologia* doi:10.1007/s00442-008-1040-z

Metges, C., Kempe, K. and Schmidt, H.-L. (1990). Dependence of the carbon-isotope contents of breath carbon dioxide, milk, serum and rumen fermentation products on the $\delta^{13}C$ value of food in dairy cows. *Brit. J. Nutr.* **63**, 187-196.

Passey, B. H., Robinson, T. F., Ayliffe, L. K., Cerling, T. H., Sponheimer, M., Dearing, M. D., Roeder, B. L. and Ehleringer, J. R. (2005). Carbon isotope fractionation between diet, breath CO_2 and bioapatite in different mammals. *J. Archaeol. Sci.* **32**, 1459-1470.

Perkins, S. E. and Speakman, J. R. (2001). Measuring natural abundance of ^{13}C in respired CO_2: variability and implications for non-invasive dietary analysis. *Funct. Ecol.* **15**, 791-797.

Podlesak, D. W., McWilliams, S. R. and Hatch, K. A. (2005). Stable isotopes in breath, blood, feces and feathers can indicate intra-individual changes in the diet of migratory songbirds. *Oecologia* **142**, 501-510.

Sponheimer, M., Robinson, T. F., Cerling, T. E., Tegland, L., Roeder, B. K., Ayliffe, L., Dearing, M. D. and Ehleringer, J. R. (2006). Turnover of stable carbon isotopes in the muscle, liver, and breath CO_2 of alpacas (*Lama pacos*). *Rapid Comm. Mass Spectr.* **20**, 1395-1399.

Tieszen, L. L. and Fagre, T. (1993). Effect of diet quality and composition on the isotopic composition of respiratory CO2, bone collagen, bioapatite, and soft tissues. In *Prehistoric Human Bone Archaeology at the Molecular Level* (ed. J. B. Lambert and G. Grupe), pp.121-155. New York: Springer Verlag.

Tieszen, L. L., Boutton, T. W., Tesdahl, K. G. and Slade, N. A. (1983). Fractionation and turnover of stable carbon isotopes in animal tissues: Implications for $\delta^{13}C$ analysis of diet. *Oecologia* **57**, 32-37.

Voigt, C. C. and Speakman, J. R. (2007). Nectar-feeding bats fuel their high metabolism directly with exogenous carbohydrates. *Funct. Ecol.* **21**, 913-921.

Voigt, C. C., Rex, K., Michener, R. H. and Speakman, J. R. (2008a). Nutrient routing in omnivorous animals tracked by stable carbon isotopes in tissue and exhaled breath. *Oecologia* doi:10.1007/s00442-008-1057-3.

Voigt, C. C., Grasse, P., Rex, K., Hetz, S. K. and Speakman, J. R. (2008b). Bat breath reveals metabolic substrate use in free-ranging vampires. *J. Comp. Physiol. B* **178**, 9-16.

Welch, K. C., Jr, Bakken, B. H., Martínez del Rio, C. and Suarez, R. K. (2006). Hummingbirds fuel hovering flight with newly ingested sugar. *Physiol. Biochem. Zool.* **79**,1082-1087.

Welch, K. C., Jr, Herrera, L. G. and Suarez, R. K. (2008). Dietary sugar as a direct fuel for flight in the nectarivorous bat, *Glossophaga soricina*. *J. Exp. Biol.* **211**, 310-316.