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Metabolic substrate use and the turnover of endogenous energy reserves in broad-tailed hummingbirds (*Selasphorus platycercus*)

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Summary

We fed broad-tailed hummingbirds (*Selasphorus platycercus*) diets of contrasting carbon isotope composition and measured changes in the $\delta^{13}C$ of expired breath through time. By measuring the $\delta^{13}C$ in the breath of fed and fasted birds we were able to quantify the fraction of metabolism fueled by assimilated sugars and endogenous energy reserves. These measurements also allowed us to estimate the fractional turnover of carbon in the hummingbirds' energy reserves. When hummingbirds were feeding, they fueled their metabolism largely (≈90%) with assimilated sugars. The rate of carbon isotope incorporation into the energy reserves of hummingbirds was higher when birds were gaining as opposed to losing body mass. The average residence time of a carbon atom in the hummingbirds' energy reserves ranged from 1 to 2 days.

Key words: $\delta^{13}C$, energy storage, fuel use, hummingbird, *Selasphorus platycercus*, isotopic incorporation, respiration, stable isotopes.

Introduction

The nutrients that animals assimilate can follow several pathways. They can be stored for future use, immediately oxidized to fuel metabolism, or used to synthesize materials for reproduction, growth and tissue maintenance. The sugars assimilated by a non-reproductive nectar-feeding bird, for example, can be stored as glycogen, used to synthesize lipid, or oxidized immediately (Alexander, 1999). Historically, studies investigating metabolic substrate use have relied on the respiratory quotient ($RQ=\dot{V}_{CO_2}\cdot\dot{V}_{O_2}^{-1}$) to assess whether carbohydrates, lipids or proteins support respiration (Surarez et al., 1990; Powers, 1991). Advances in elemental stable isotope analysis now allow an alternative/complementary method to RQ to determine metabolic substrate use. Recently, stable isotopes have been used to clarify nutritional, physiological and ecological questions that respirometry could not, for instance, to quantify the relative contribution of ingested ('income') to stored ('capital') nutrients for reproduction in adults of both moths and butterflies (O'Brien et al., 2000; O'Brien et al., 2004). Stable isotopes have also been used in migratory birds to discriminate between nutrients ingested on the wintering grounds and during migration *versus* those ingested on the breeding grounds (Hobson et al., 2000). These studies relied on animals that either naturally or artificially switched between diets of distinct isotopic composition.

We used a similar diet-shifting approach to clarify metabolic substrate use in hummingbirds. Carleton et al. found that the carbon stable isotope composition ($\delta^{13}C$) of respired CO_2 from feeding rufous hummingbirds (*Selasphorus rufus*) closely resembled that of dietary nutrients (Carleton et al., 2004). When they switched birds to a diet with a contrasting isotopic composition, $\delta^{13}C$ of respired CO_2 was intermediate between diets, which indicated that hummingbirds were metabolizing both exogenous nutrients and endogenous reserves. Here, by measuring $\delta^{13}C$ of exhaled CO_2 in animals that were shifted between diets with contrasting carbon isotope compositions, we were able to quantify the fraction of metabolism fueled by income (assimilated sugars) and capital (endogenous reserves) in broad-tailed hummingbirds (*Selasphorus platycercus* Swainson). Additionally, because stable isotopes allow determining isotopic incorporation of assimilated nutrients into an organism's tissues (Carleton et al., 2004), we examined both the isotopic incorporation of carbon and the mean residence time of a carbon atom in the endogenous reserves of broad-tailed hummingbirds.

Materials and methods

Hummingbird maintenance and experimental design

We captured male broad-tailed hummingbirds *Selasphorus platycercus* Swainson (N=8) with mist-nets in Albany County, Wyoming, USA (41°20′N, 106°15′W). Birds were housed under a 15 h:9 h photoperiod (photophase: 05:30 h–20:30 h MST) in a room at 20±2°C. Hummingbirds fed *ad libitum* on a diet derived from C_3 plants (Nektar-Plus®, Guenter Enderle, Tarpon Springs, FL, USA; $\delta^{13}C$=–24.2±0.09, N=10) for ≈90

days prior to experiments. This was to ensure that the $\delta^{13}C$ of their endogenous reserves reflected that of the C_3-derived diet (see List of abbreviations and symbols). Our experiment had three phases. During phase 1 (day –11 to –1), we verified on three dates (day –11, –8 and –4) that birds were in isotopic equilibrium with their C_3 diet (Fig. 1). During phase 2 (day 0 to 19), birds fed *ad libitum* on a 20% (mass percent) sucrose solution derived from C_4 plants ($\delta^{13}C$=–11.4±0.07, N=10) and fruit flies ($\delta^{13}C$=–23.0±0.4, N=11). In phase 3 (day 20 to 43), birds were shifted back to the C_3-derived diet. We weighed birds (at 08:30 h–09:00 h) periodically and measured their food intake throughout the experiment.

For phases 1, 2 and 3, we measured the $\delta^{13}C$ in the breath of birds after an overnight fast ('fasted') at 05:30 h and on birds that had *ad libitum* access to food ('fed') at 12:00 h. Briefly, birds were taken from their cage, lightly restrained within a sleeve of laboratory tissue and introduced into 50 ml polypropylene centrifuge tube. This tube had an internal diameter of 28 mm and was fitted with a one-way stopcock valve (Fig. 1). After introducing the bird in the tube, we gently flushed the tube with ≈500 ml of CO_2-free air over a 30 s period. Tubes were then sealed and exhaled CO_2 was allowed to accumulate for 1 min. A 30 ml air sample was then extracted using a gastight syringe. Withdrawing the gas causes a sudden change in the pressure inside the tube. To avoid injuring the birds we immediately re-pressurized the chamber following gas extraction by opening the stopcock valve. Birds were unaffected by this procedure. Samples were gathered within 3 min after birds were taken from their cages. We injected air samples into pre-evacuated gastight vials (Exetainer®; Labco Ltd, Buckinghamshire, UK) until a positive pressure was achieved.

Stable isotope analyses

We measured the isotopic composition of expired CO_2 on a Micromass VG Optima continuous flow mass spectrometer coupled to a micro gas injector (GV Instruments, Manchester, UK) at the Mass Spectrometry Isotope Facility, Colorado State University (Fort Collins, CO, USA). The precision of these analyses was ±0.2‰ and our standard was gaseous CO_2 ($\delta^{13}C$=–37.8‰, VPDB). Our method is similar to that developed by Hatch et al. (Hatch et al., 2002) and applied by Podlesak et al. (Podlesak et al., 2005), except that we did not use party balloons.

Carbon isotope ratios of food were measured on a continuous flow isotope ratio mass spectrometer with samples combusted in a Carlo Erba NA 1500 elemental analyzer (Milan, IT). The precision of these analyses was ±0.2‰. Our standards were vacuum oil ($\delta^{13}C$=–27.5‰, VPDB) and Australian National University sucrose ($\delta^{13}C$=–10.5‰, VPDB, NIST 8542). We included standards in every run to correct raw values obtained from the mass spectrometer. Isotope ratios in this paper are reported as δ values on a per mil (‰) basis relative to the International Atomic Energy Agency carbon isotope standard, Vienna Pee Dee Belemnite (VPDB).

Modeling and statistical analyses

To compare among energy ingestion rates during the three experimental phases, we used repeated-measures analysis of variance (RM-ANOVA) and Tukey's Honest Significant

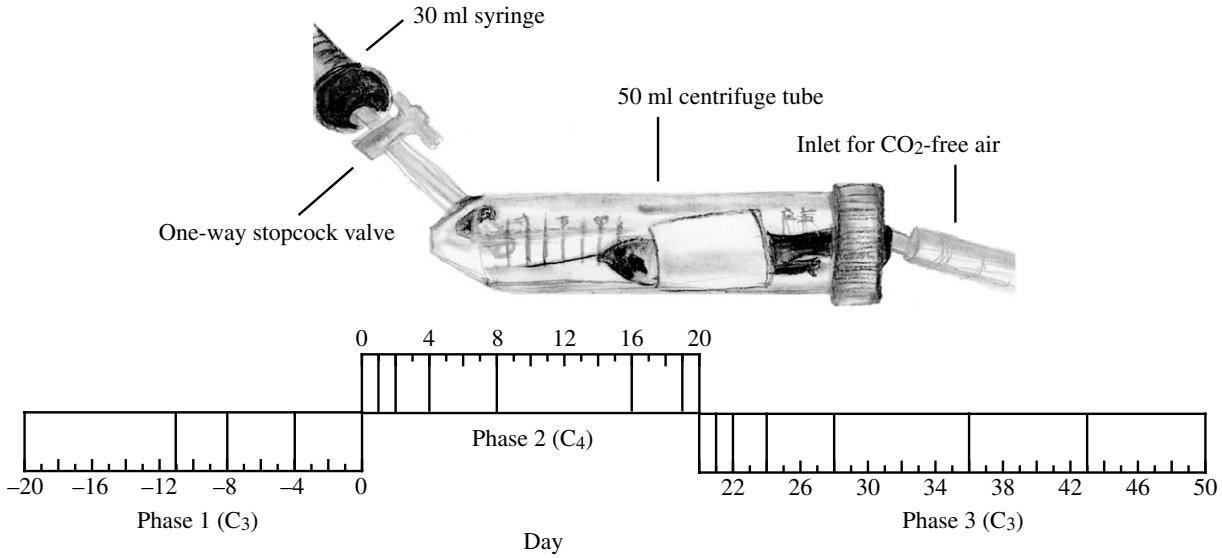

Fig. 1. Experiments consisted of three phases. In phase 1, broad-tailed hummingbirds (*Selasphorus platycercus*) were fed on a C_3 diet for roughly 3 months. Phase 2 began on day 0 when the isotopic composition of the diet was changed from C_3 to C_4. Phase 2 lasted 20 days. Phase 3 began on day 20, when the isotopic composition of the hummingbirds' diet was shifted back to the original C_3. Vertical lines denote the days we measured $\delta^{13}C$ of exhaled CO_2 in fasted and fed birds. We collected exhaled air from hummingbirds that were lightly restrained within a centrifuge tube that had been previously flushed with CO_2-free air.

Difference tests to compare among means. We compared between the $\delta^{13}C$ values of fasted and fed birds using paired *t*-tests. We estimated the fractional rate of isotopic incorporation (k) with a non-linear fitting procedure for each individual bird using the equation:

$$\delta^{13}C(t) = \delta^{13}C(\infty) + [\delta^{13}C(0) - \delta^{13}C(\infty)]e^{-kt}, \quad (1)$$

where $\delta^{13}C(t)$ is the isotope composition at time *t*, $\delta^{13}C(0)$ is the estimated initial isotope composition, $\delta^{13}C(\infty)$ is the asymptotic equilibrium isotope composition and k is the fractional rate of isotope incorporation (O'Brien et al., 2000; Carleton and Martínez del Rio, 2005). Eqn 1 assumes that the incorporation of carbon into energy reserves follows single-compartment, first-order kinetics. The reciprocal of the fractional rate of isotopic incorporation (k^{-1}) estimates the average residence time (days) of a carbon atom in energy reserves. We compared the fractional rates of isotopic incorporation between phases 2 and 3 with paired *t*-tests. We calculated the fractionation between breath and diet using the equation:

$$\Delta\delta^{13}C = \delta^{13}C_{tissue(\infty)} - \delta^{13}C_{diet}. \quad (2)$$

We used standard least squares linear regression to estimate rates of change in body mass. Unless noted to the contrary, N=8 for our analyses. We report data as means ± s.d.

Results

Energy consumption and body mass

Birds ingested significantly different quantities of energy during the three phases of our experiment (RM-ANOVA: $F_{2,6}$=6.6, P=0.03). A Tukey's *post hoc* test, however, revealed that energy consumption was only greater in phase 1 (C_3; 25.9±2.5 kJ day^{-1}); birds had similar energy consumption rates in phases 2 (C_4; 23.8±1.8 kJ day^{-1}) and 3 (C_3; 23.5±2.3 kJ day^{-1}). Birds maintained body mass in phase 1 and lost mass at a slow rate in phase 2 (0.02±0.01 g day^{-1}, mean $r_{mass,time}$=–0.85, P<0.001; Fig. 2). However, they regained mass at a constant rate during the first 10 days of phase 3 (0.04±0.02 g day^{-1}, mean $r_{mass,time}$=0.84, P<0.001), after which they maintained relatively constant body mass (Fig. 2).

Isotopic composition of expired breath

During phase 1, the birds breath had a distinctly C_3 signature. The exhaled CO_2 of birds when fasted overnight was significantly more negative ($\delta^{13}C$=–27.1±0.2) than after 6 h of feeding ($\delta^{13}C$=–25.5±0.2; paired *t*-test: t_7=19.2, P<0.001; Fig. 3). On the days of a diet shift (day 0 and 20), the $\delta^{13}C$ in exhaled CO_2 of fed and fasted birds was dramatically different (Fig. 3). Fasted birds exhaled CO_2 with $\delta^{13}C$ that closely resembled that of their previous diet, whereas fed birds exhaled CO_2 with $\delta^{13}C$ that closely resembled that of the new diet (Fig. 3). On day 0, fasted and fed birds exhaled CO_2 with $\delta^{13}C$= –26.6±0.6 and –13.2±1.1‰, respectively (paired *t*-test: t_7=37.5, P<0.001); on day 20, fasted and fed birds exhaled CO_2 with $\delta^{13}C$=–13.1±0.6 and –23.2±1.2‰, respectively (paired *t*-test: t_7=25.9, P<0.001).

On a diet-shift day, the $\delta^{13}C$ of breath in fed birds was very similar to that of their diet; however, these two values were not identical. On day 0, $\delta^{13}C$ of breath in fed birds was slightly, but significantly, more negative than that of their diet (one sample *t*-test: t_7=4.5, P=0.003; Fig. 3); on day 20, $\delta^{13}C$ of breath in fed birds was slightly, but significantly, more positive than that of their diet (one sample *t*-test: t_7=2.5, P=0.04; Fig. 3). During phases 2 and 3, the $\delta^{13}C$ of fed birds' breath changed through time and eventually came to resemble that of their current diet (Fig. 3).

Rates of isotopic incorporation

The change in $\delta^{13}C$ of CO_2 exhaled by fasted birds was well described by Eqn 1 (the coefficients of determination ranged from 0.89 to 0.97; Fig. 3). The fractional rate of isotopic incorporation, k, was significantly higher in phase 3 than in phase 2 (k=0.86±0.16 and 0.47±0.19, respectively; Table 1) and the asymptotic carbon isotopic composition of the breath [$\delta^{13}C(\infty)$] of fasted birds was significantly more depleted in ^{13}C than that of their food (one sample *t*-tests: phase 1, t_7=14.4, P<0.0001; phase 2, t_7=24.2, P<0.0001; Fig. 1, Table 1).

Discussion

In our discussion, we compare the findings of our stable

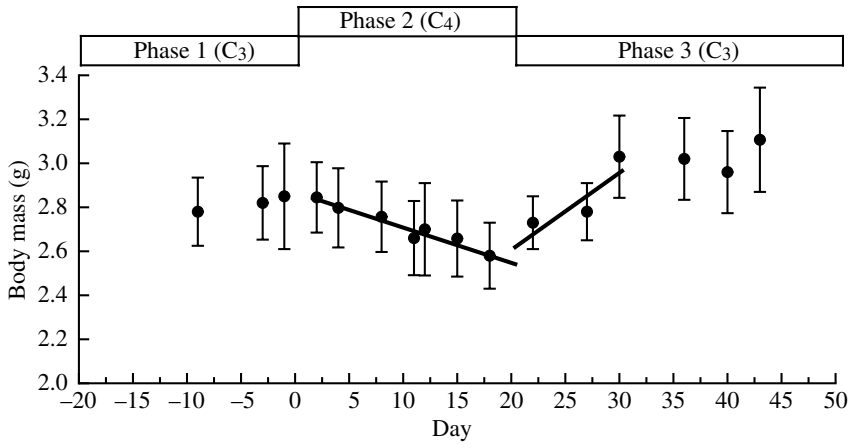

Fig. 2. Broad-tailed hummingbirds (*Selasphorus platycercus*) maintained mass in phase 1, but lost mass in phase 2. Birds regained mass during the first 10 days of phase 3. Regression lines were drafted from the averages of the intercepts and slopes of the relationships between body mass and time for individual birds. The coefficients of correlation of these relationships were significantly negative in phase 2 (r=–0.85, t_7=17.8, P<0.001) and significantly positive in the first 10 days of phase 3 (r=0.81, t_7=22.2, P<0.001). The slopes of these relationships (the rate of daily mass change) are reported in the text.

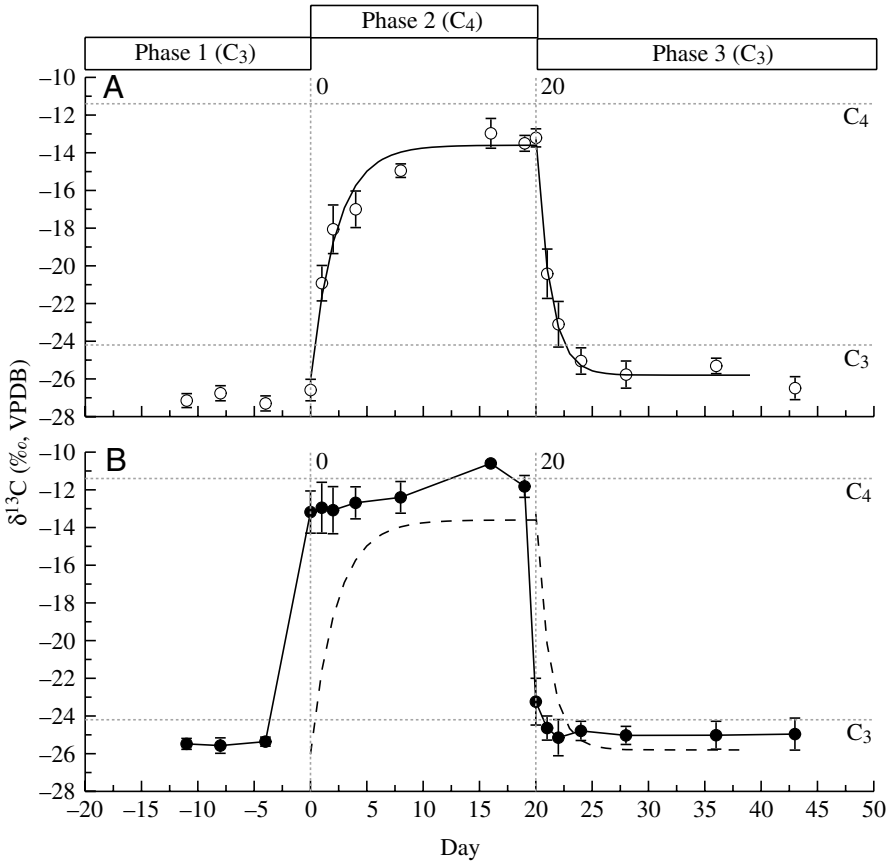

Fig. 3. $\delta^{13}C$ of expired breath in fasted and fed broad-tailed hummingbirds (*Selasphorus platycercus*). The dotted gray lines parallel to the *x*- and *y*-axes denote the $\delta^{13}C$ of diets and diet shift days, respectively. (A) After the carbon isotopic composition of the diet was changed from C_3 to C_4 (day 0 to 19), the $\delta^{13}C$ of exhaled CO_2 in fasted birds changed following one-compartment, first order kinetics with a fractional rate of isotopic incorporation equal to 0.47±0.19 day^{-1}. When the isotopic composition of the hummingbirds' diet was changed from C_4 to C_3 (day 20 to 43), the $\delta^{13}C$ of exhaled CO_2 in fasted birds changed with a faster fractional rate of isotopic incorporation (0.86±0.16 day^{-1}). Note that the asymptotic value of $\delta^{13}C$ in the fasted hummingbirds' breath was more negative than that of their diet (represented by dotted horizontal lines). We constructed these curves using the parameters in Table 1. (B) Immediately after a diet shift, the $\delta^{13}C$ of breath in fed birds changed to resemble closely (but not exactly) that of the new diet. The broken curve is the value predicted for fasted birds and is presented to compare the differences between measurements done on fed and fasted birds.

isotope approach to those of respirometric studies on metabolic substrate usage in hummingbirds. We then use $\delta^{13}C$ of expired breath to quantify the fraction of metabolism fueled by endogenous and exogenous nutrients. We also estimate isotopic incorporation rates and carbon atom residence times in hummingbirds, and consider how energy balance affects them. We conclude our discussion by addressing the possibilities and limitations of our stable isotope approach to ecological studies.

Do hummingbirds fuel metabolism with income or capital?

On the days that birds shifted diets (day 0 and 20), fasted birds exhaled CO_2 with $\delta^{13}C$ that resembled that of their previous diet, whereas fed birds exhaled CO_2 with $\delta^{13}C$ that closely resembled that of the new diet (Fig. 3). These results support the notion that fed hummingbirds fuel their metabolism primarily with recently ingested sugars, whereas fasted hummingbirds use endogenous reserves (Suarez et al., 1990). Our results are consistent with measurements on Anna's (*Calypte anna*) and Costa's (*Calypte costae*) hummingbirds (Powers, 1991). During the day, when birds were feeding, Powers found that their RQ was approximately 1.0, which indicates that birds were oxidizing sugars; after an overnight fast, their RQ was close to 0.7, which indicates that birds were oxidizing lipids (Powers, 1991). Although our stable isotope approach does not allow identifying the endogenous substrates used by fasted hummingbirds, the significant difference between the

Table 1. *Isotopic incorporation rates in broad-tailed hummingbirds (Selasphorus platycercus)*

	k (day^{-1})	$\delta^{13}C(0)$	$\delta^{13}C(\infty)$	$\Delta^{13}C_{breath\text{-}food}$
Phase 2 (C_4)	0.47±0.19	–25.89±0.28	–13.7±0.5	–2.3±0.5
Phase 3 (C_3)	0.86±0.16	–13.17±0.59	–25.5±0.5	–1.6±0.6

The fractional rate of isotopic incorporation (k) was significantly higher in phase 3 (C_3) compared to phase 2 (C_4; paired *t*-test: t_7=8.1, P<0.001). The asymptotic carbon isotopic composition in the breath of fasted birds [$\delta^{13}C(\infty)$] was similar to that of their diet, but it was significantly more depleted in ^{13}C. Thus, the average discrimination factor between diet and breath ($\Delta^{13}C_{breath\text{-}food}$) was significantly different from 0 (one sample *t*-tests: t_7>14.4, P<0.0001).

We obtained the parameters in this table by fitting the equation $\delta^{13}C(t)=\delta^{13}C(\infty) + [\delta^{13}C(0)–\delta^{13}C(\infty)]e^{–kt}$ to data for individual birds (N=8) using a non-linear fitting routine. Values are means ± s.d.; C_3, C_4, and other symbols and abbreviations are defined in the List of abbreviations and symbols.

$\delta^{13}C$ of food and breath in birds in isotopic equilibrium suggests that a large fraction of the substrates oxidized by fasted hummingbirds were lipids (Table 1). In general, lipids are depleted in ^{13}C relative to the carbohydrates from which they are synthesized (DeNiro and Epstein, 1977). Our hypothesis, that endogenous lipids fuel the fasting metabolism of hummingbirds, can be tested by measuring $\delta^{13}C$ in expired breath and RQ concurrently.

Our results are also consistent with the predictions of Suarez et al. (Suarez et al., 1990), who proposed that active, fed hummingbirds should oxidize carbohydrates preferentially to fuel respiration and rapidly shift to lipids after even very short fasts (Suarez and Gass, 2002). Using dietary sugars as fuel when feeding is advantageous because using synthesized fat to fuel respiration entails an approximately 16% cost of synthesis. However, hummingbirds are small and have high metabolic rates. Thus, in order to spare their small glycogen reserves, they must shift to the oxidation of lipids even after short fasts (Suarez and Gass, 2002). Changes in the $\delta^{13}C$ in breath of fasting hummingbirds can reveal the details of shifts in substrate oxidation during the transition from the absorptive to the postabsorptive state.

Although the $\delta^{13}C$ of fed birds closely resembled that of the new diet following a diet shift, it was not identical to it (Fig. 3). One interpretation is that, although hummingbirds oxidized mostly carbohydrates, they also oxidized a small fraction of endogenous reserves. This interpretation is strengthened by the decreasing difference between the $\delta^{13}C$ of the breath of fed birds and that of diet as the isotope composition of endogenous reserves came to resemble that of the new diet following a diet shift (Fig. 3). A linear mixing model can be used to estimate the fraction of endogenous substrates oxidized by fed hummingbirds (Carleton et al., 2004). This model estimates the fraction (P) contributed by endogenous reserves, with an isotope composition equal to $\delta^{13}C_{fasted}$, relative to the fraction (1–P) contributed by dietary sugars, with an isotope composition equal to $\delta^{13}C_{diet}$, so that:

$$P = \frac{\delta^{13}C_{fed} - \delta^{13}C_{diet}}{\delta^{13}C_{fasted} - \delta^{13}C_{diet}} \quad . \qquad (3)$$

We only estimated P for day 0 and 20 because here the end-points of the mixing model were sufficiently distinct to allow using Eqn 2 with confidence. Endogenous reserves contributed 11.6±7.3 and 8.5±11.0% (paired *t*-test: t_7=0.7, P>0.5) to total respiration on day 0 and 20, respectively. Although fed hummingbirds fueled respiration primarily (≈90%) with dietary sugars, they oxidized a small fraction of endogenous reserves as well (Carleton et al., 2004). Surprisingly, during phase 2, there was no evidence of a significant contribution of the isotope composition of fruit flies in the $\delta^{13}C$ of breath of hummingbirds. We hypothesize that hummingbirds routed the protein contained in this component of their diets directly into the manufacture of body protein and thus spared the protein in fruit flies from oxidation (Martínez del Rio and Wolf, 2005).

Hummingbird energy reserves have remarkably high carbon fluxes

Hummingbirds incorporated the isotope signal of their diet into endogenous reserves very rapidly (Table 1). The average residence time of a carbon atom in a hummingbird's endogenous reserves can be estimated as k^{-1}. On average, between assimilation, storage and oxidation, a dietary carbon atom remained in a hummingbird's energy reserves only between 1 and 2 days. The remarkably high mass-specific metabolic rates of hummingbirds (Suarez and Gass, 2002) explain their high rates of isotope incorporation, and hence the high rates of carbon flux, into energy reserves. Carpenter et al. estimated that non-migrating hummingbirds store between 0.2 and 0.5 g of lipids (Carpenter et al., 1993). Assuming endogenous reserves comprise primarily lipids, hummingbirds turned over 0.10 to 0.24 g lipid day^{-1} in phase 2, when they were losing body mass. These numbers are within the range of lipid masses lost overnight by congeneric rufous hummingbirds (Carpenter et al., 1993).

Effect of mass changes on the rate of isotopic incorporation

A serendipitous result of our experiment allowed us to address how isotopic incorporation is affected by energy balance. The fractional rate of isotope incorporation (k) was almost twice as high in phase 3 compared to phase 2 (Fig. 3). This disparity has a relatively straightforward explanation. Birds were losing body mass, presumably including endogenous energy reserves, during phase 2, but gaining it during phase 3. Fry and Arnold (Fry and Arnold, 1982) and Hesslein et al. (Hesslein et al., 1993) recognized that the value of k is determined by both the addition of new material ('net growth') and by the replacement of material exported from the tissue as a result of catabolism ('catabolic turnover'). If the animal is losing endogenous reserves, k equals the fraction of new materials from the diet that partially replace the materials lost by catabolism. However, if the animal is increasing the size of its endogenous reserves, then k has two components: the fractional rate of storage, which represents a net addition to endogenous reserves, and the fractional rate of oxidation, which represents replacement. Therefore, an increase in the size of endogenous reserves equates to higher fractional rates of isotope incorporation.

Ecological implications

Stable isotopes have been used to investigate what animals eat (Dalerum and Angebörn, 2005) and to assess the temporal variation in their diets (Hatch et al., 2002). A particularly informative approach is to use tissues that incorporate dietary isotope signatures at different rates within a single individual (Podlesak et al., 2005; Dalerum and Angerbörn, 2005). Our experiment established that fed hummingbirds oxidized primarily, albeit not exclusively, dietary sugars. Thus, the carbon isotope composition of breath in a fed hummingbird provided a snapshot of the isotopic composition of what the animal was eating. Our experiments also allowed us to measure the turnover of endogenous reserves and revealed it was brisk.

Therefore, we were able to use the $\delta^{13}C$ values in breath to distinguish between what animals were eating currently and what they ate previously, but only on the days of a diet shift (day 0 and 20). Two days after a diet shift, the CO_2 of the breath of fasted birds contained a mixture of carbon from the old and the new diet. Hummingbirds incorporate dietary carbon into their energy reserves so rapidly that the $\delta^{13}C$ in the breath of fed and fasted animals cannot be used to assess temporal variation in the isotope composition of their diet – except at very short time scales. Carleton and Martínez del Rio demonstrated that the rate of isotopic incorporation into blood is an allometric function of body mass (Carleton and Martínez del Rio, 2005). Therefore, we expect that larger animals will have slower carbon fluxes into their energy reserves. Measuring $\delta^{13}C$ in absorptive and postabsorptive individuals of larger species will likely resolve temporal variation in diet over longer time scales (Hatch et al., 2002). However, measuring $\delta^{13}C$ in absorptive and postabsorptive animals to resolve temporal variation in the isotopic composition of diet will require determining the allometric relationship between carbon atom residence time in energy reserves and body mass.

List of abbreviations and symbols

$\delta^{13}C$	$[(^{13}C/^{12}C_{sample}-^{13}C/^{12}C_{standard})^{13}C/^{12}C_{standard}^{-1}] \times 10^3$ (‰, VPDB)
$\delta^{13}C_{diet}$	isotope composition of the diet (‰, VPDB)
$\delta^{13}C_{tissue}$	isotope composition of the tissue (‰, VPDB)
$\delta^{13}C_{fasted}$	isotope composition of breath from a fasted bird (‰, VPDB)
$\delta^{13}C_{fed}$	isotope composition of breath from a fed bird (‰, VPDB)
$\delta^{13}C(\infty)$	asymptotic equilibrium isotope composition (‰, VPDB)
$\delta^{13}C(0)$	initial isotope composition (‰, VPDB)
$\delta^{13}C(t)$	isotope composition at time t (‰, VPDB)
^{12}C	carbon isotope (6 protons, 6 neutrons) (moles)
^{13}C	carbon isotope (6 protons, 7 neutrons) (moles)
C_3	C_3 photosynthetic pathway
C_4	C_4 photosynthetic pathway
k	fractional rate of isotopic incorporation (day^{-1})
k^{-1}	residence time (days)
MST	mountain standard time (24 h)
NIST	National Institute of Standards and Technology
P	proportion
RM-ANOVA	repeated-measures analysis of variance
RQ	respiratory quotient (dimensionless)
t	time (days)
$\dot{V}_{CO_2}$	carbon dioxide production rate (ml min^{-1})
$\dot{V}_{O_2}$	oxygen consumption rate (ml min^{-1})
VPDB	Vienna Pee Dee Belemnite

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References

Alexander, R. M. (1999). *Energy for Animal Life*. Oxford: Oxford University Press.

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., Wolf, B. O. and Martínez del Rio, C. (2004). Keeling plots for hummingbirds: a method to estimate carbon isotope ratios of respired CO_2 in small vertebrates. *Oecologia* **141**, 1-6.

Carpenter, F. L., Hixon, M. A., Beuchat, C. A., Russell, R. W. and Paton, D. C. (1993). Biphasic mass gain in migrant hummingbirds: Body composition changes, torpor, and ecological signifcance. *Ecology* **74**, 1173-1182.

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.

Fry, B. and Arnold, C. (1982). Rapid $^{13}C/^{12}C$ turnover during growth of brown shrimp (*Penaeus aztecus*). *Oecologia* **54**, 200-204.

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

Hesslein, R. H., Hallard, K. A. and Ramlal, P. (1993). Replacement of sulfur, carbon, and nitrogen in tissue of growing broad whitefish (*Coregonus nasu*) in response to a change in diet traced by ^{34}S, ^{13}C, and ^{15}N. *Can. J. Fish. Aquat. Sci.* **50**, 2071-2076.

Hobson, K. A., Sirois, J. and Gloutney, M. L. (2000). Tracing nutrient allocation to reproduction with stable isotopes: a preliminary investigation using colonial waterbirds of Great Slave Lake. *Auk* **117**, 760-774.

Martínez del Rio, C. and Wolf, B. O. (2005). Mass balance models for animal isotopic ecology. In *Physiological and Ecological Adaptation to Feeding in Vertebrates* (ed. J. M. Starck and T. Wang), pp. 141-174. New Hampshire: Science Publishers.

O'Brien, D., Schrag, D. and Martínez del Rio, C. (2000). Allocation of nectar nutrients to reproduction in *Amphion floridensis*: a novel quantitative method using stable isotopes. *Ecology* **81**, 2822-2831.

O'Brien, D. M., Boggs, C. L. and Fogel, M. L. (2004). Making eggs from nectar: the role of life history and dietary carbon turnover in butterfly reproductive resource allocation. *Oikos* **105**, 279-291.

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.

Powers, D. R. (1991). Diurnal variation in mass, metabolic rate, and respiratory quotient in Anna's and Costa's hummingbirds. *Physiol. Zool*. **64**, 850-870.

Suarez, R. K. and Gass, C. L. (2002). Hummingbird foraging and the relation between bioenergetics and behaviour. *Comp. Biochem. Physiol.* **133A**, 225-343.

Suarez, R. K., Lighton, J. R. B., Moyes, C. D., Brown, G. S., Gass, C. L. and Hochachka, P. W. (1990). Fuel selection in rufous hummingbirds: ecological implications of metabolic biochemistry. *Proc. Natl. Acad. Sci. USA* **87**, 9207-9210.