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METHODS

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# Keeling plots for hummingbirds: a method to estimate carbon isotope ratios of respired $CO_2$ in small vertebrates

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**Abstract** The carbon isotope composition of an animal's breath reveals the composition of the nutrients that it catabolizes for energy. Here we describe the use of Keeling plots, a method widely applied in ecosystem ecology, to measure the $\delta^{13}C$ of respired $CO_2$ of small vertebrates. We measured the $\delta^{13}C$ of Rufous Hummingbirds (*Selasphorus rufus*) in the laboratory and of Mourning (*Zenaida macroura*) and White-winged (*Z. asiatica*) Doves in the field. In the laboratory, when hummingbirds were fed a sucrose based C3 diet, the $\delta^{13}C$ of respired $CO_2$ was not significantly different from that of their diet ($\delta^{13}C_{C3\ diet}$). The $\delta^{13}C$ of respired $CO_2$ for C3 fasted birds was slightly, albeit significantly, depleted in $\delta^{13}C$ relative to $\delta^{13}C_{C3\ diet}$. Six hours after birds were shifted to a sucrose based C4 diet, the isotopic composition of their breath revealed that birds were catabolizing a mixture of nutrients derived from both the C3 and the C4 diet. In the field, the $\delta^{13}C$ of respired $CO_2$ from Mourning and White-winged Doves reflected that of their diets: the CAM saguaro cactus (*Carnegeia gigantea*) and C3 seeds, respectively. Keeling plots are an easy, effective and inexpensive method to measure $\delta^{13}C$ of respired $CO_2$ in the lab and the field.

**Keywords** *Selasphorus rufous* · *Zenaida asiatica* · $\delta^{13}C$ · Keeling plots · Diet reconstruction

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## Introduction

The number of publications in animal ecology and physiological ecology that use stable isotopes has doubled every 3 years over the last 10 years (Martínez del Rio and Wolf 2003). This is an extraordinarily high rate of increase for the incorporation of any scientific methodology. Stable isotope analyses are versatile tools and their versatility is one of the reasons for their usefulness. Natural ratios of stable isotope have been used to determine patterns of resource allocation to reproduction (Hobson 1995; Obrien et al. 2002), to track animal migration (Hobson 1999), to assess the flux of materials from the sea into terrestrial food webs (Hobson and Sealy 1991; Ben David et al. 1998), to assign trophic levels (Post 2002), and to determine the structure of food webs (France 1995). Most of these applications rely on the observation that the isotopic composition of animal tissues reflects that of the nutrients that they assimilate (DeNiro and Epstein 1978; Hobson and Clark 1992a, 1992b).

When an animal shifts between diets with contrasting isotopic compositions, the isotopic composition of the new diet is not incorporated instantaneously into all tissues. Some tissues incorporate isotopes faster than others (Tieszen et al. 1983; Hobson and Clark 1992a, 1992b; Bearhop et al. 2002). This variation in the time-course of isotopic incorporation is both advantageous and challenging. It is advantageous because it allows reconstructing diets at different time scales from different tissues. It is challenging because to interpret temporal changes in the isotopic composition of animal tissues, we need to know the rate at which isotopes are incorporated into different tissues. Some tissues, such as liver and plasma proteins, have high turnover rates, and their isotopic composition reflects integration of recent inputs in the order of days to weeks (Hobson and Clark 1992a). Other tissues, such as red blood cells, muscle, and bone collagen, have slower turnover rates and their isotopic compositions reflect integration of dietary inputs over longer time periods. Tissues with high nutrient turnover rates track isotopic changes in diet closely, whereas tissues with low nutrient

turnover rates integrate isotope diet signatures from a large temporal window. If the question is to determine how animals track the availability of resources, then a tissue with high turnover rate (i.e. plasma proteins and liver) must be used.

The carbon isotope composition of the $CO_2$ exhaled by an animal is believed to represent the $\delta^{13}C$ of the substrate catabolized at the time of measurement (Schoeller et al. 1980, 1984; Metges et al. 1990; Normand et al. 1992; Hatch et al. 2002a, 2002b). It is considered the closest that we can get to an instantaneous snapshot of the isotope composition of an animal's diet (Hatch et al. 2002a). Although measuring the isotopic composition of respired $CO_2$ is potentially informative, it is rarely done. The paucity of measurements is to some extent the result of the difficulties involved in measuring the isotopic composition of exhaled $CO_2$ in the field. Here we demonstrate that the same methods used to estimate the isotope composition of an ecosystem's respiration can be used to estimate that of a small animal in the laboratory and field.

Plant ecosystems ecologists use Keeling plots (Keeling 1958, 1961) to measure the isotopic composition of $CO_2$ produced by the vegetation and soil in whole ecosystems (Flanagan and Ehleringer 1997; Yakir and Sternberg 2000). Because the concentration of $CO_2$ increases at night as a result of respiration, it causes a decline in the $^{13}C/^{12}C$ ratio of the air within the forest boundary layer (Dawson et al. 2002). The co-variation in $\delta^{13}C$ and the concentration of $CO_2$ within this layer can be used to estimate the $^{13}C/^{12}C$ ratios of the forest's respiration. In a Keeling plot, the $\delta^{13}C$ of air within a vegetation canopy is plotted against the inverse of the concentration of $CO_2$ [$1/(CO_2)_{system}$]. The *y*-intercept of this linear relationship estimates the carbon isotope composition of the ecosystem's respiration (see Eq. 2).

Keeling plots assume that the carbon isotope composition of atmospheric $CO_2$ within a vegetation canopy reflects the mixture of some background amount of $CO_2$ that is already present in the atmosphere and some amount of $CO_2$ that is added by sources in the ecosystem (Dawson et al. 2002). The equation that summarizes this isotopic mass balance is:

$$\delta^{13}C_{system}[CO_2]_{system} = \delta^{13}C_{atm}[CO_2]_{atm} + \delta^{13}C_{source}[CO_2]_{source} \quad (1)$$

where $\delta^{13}C_{system}$, $\delta^{13}C_{atm}$, and $\delta^{13}C_{source}$ are the carbon isotope values for the ecosystem, the background atmosphere, and the source, respectively (Yakir and Sternberg 2000). The concentrations of $CO_2$ contributed by each of these components are given by $[CO_2]_{system}$, $[CO_2]_{atm}$, and $[CO_2]_{source}$, respectively. Because of mass-balance, $[CO_2]_{system} = [CO_2]_{atm} + [CO_2]_{source}$ and therefore Eq. 1 can be re-arranged to give:

$$\delta^{13}C_{system} = [CO_2]_{atm} \times (\delta^{13}C_{atm} - \delta^{13}C_{source}) \times \frac{1}{[CO_2]_{system}} + \delta^{13}C_{source}. \quad (2)$$

The same principles used by ecosystem scientists can be applied to measure the carbon isotope composition in an animal's breath. Imagine that you place an animal into a closed container. At first, the isotopic composition of the $CO_2$ within the container resembles that of background atmospheric air [360 ppm, −7.8‰ Vienna Pee Dee Belemnite (VPDB)]. However, as the animal respires, the concentration of $CO_2$ in the container increases and its carbon isotope composition begins to trend towards that of the animal's respired $CO_2$. The *y*-intercept of a linear plot of $\delta^{13}C$ against $[CO_2]^{-1}$ estimates the carbon isotope ratio of the animal's respiration (Eq. 2). We used Keeling plots to measure the $\delta^{13}C$ of respired $CO_2$ in Rufous Hummingbirds (*Selasphorus rufus*) fed on diets of contrasting carbon isotope composition in the laboratory. We also applied the method to White-winged Doves (*Zenaida asiatica*) and Mourning Doves (*Z. macroura*) in the field. Isotopic ratios in this paper are reported on a per mil (‰) basis relative to the IAEA carbon isotope standard VPDB.

## Materials and methods

### Laboratory measurements

Six Rufous Hummingbirds captured in Albany County, Wyoming, were fed for 3 months on a C3 plant-based maintenance diet with $\delta^{13}C$ equal to −25.1±0.1‰ VPDB, *n*=5 (Nekton+, Guenter Enderle Enterprises, 27 West Tarpon Avenue, Tarpon Springs, Fla. 34689, USA). We measured the $\delta^{13}C$ in exhaled $CO_2$ of the birds fed this diet after an overnight fast and again after 6 h of feeding. Two days later we again measured the $\delta^{13}C$ in exhaled $CO_2$ of the birds fed this diet after an overnight fast and after they fed for 6 h on a new diet with $\delta^{13}C$=−11±0.1‰ VPDB. Measurements on fasted birds were within 15 min of the time at which lights were turned on. Birds had no access to food either overnight or during the brief period with lights on before measurements. Measurements on fed birds were conducted 6 h after lights were turned on.

Hummingbird diets consist primarily of dilute (≈20% wt/volume) sugar solutions with trace amounts of electrolytes and amino acids (Baker and Baker 1983). The carbon isotope composition of our experimental diets reflected the composition of the sugars within them. The maintenance diet contained sucrose derived from sugar beet (a C3 plant), whereas the second diet contained sucrose derived from sugar cane (a C4 plant). For brevity, we will refer to these diets as C3 and C4, respectively.

During measurements, we immobilized birds with a snug tube of laboratory tissue paper (Kim Wipe). To minimize variation among birds, measurements on fed and fasted birds were done on the same day. All laboratory

measurements were conducted in September 2002. Each bird was placed within a 1.16-l Tupperware container whose lid was fitted with a one-way stopcock valve. The stop-cock valve was connected to a tight 7-mm BEV-Line sampling tube that entered 5 cm into the chamber. Lids also had a 4-mm air hole for minimal ventilation. Air from these containers was drawn with a 30-ml syringe after pumping the syringe 3 times to increase mixing of air within the container. After the sample was withdrawn, the syringe was disconnected from the chamber tubing and a chromatography needle was attached to the syringe. The sample was then injected into a pre-evacuated 15-ml Exetainer Vacutainer (LabConCo) until a positive pressure was obtained. $CO_2$ in air samples were taken at 4, 8, 12, 16, 20, and 24 min after a bird was placed in the container.

Air samples were analyzed for $^{13}C/^{12}C$ ratios at the Mass Spectrometry Isotope Facility at Colorado State University. $\delta^{13}C$ ratios were measured on a Micromass VG Optima continuous flow mass spectrometer coupled to a micro gas injector. Sample injections were made with calibrated gastight syringes and were varied from 100 to 300 µl depending on sample $CO_2$ concentration in order to generate output voltage peaks in the 2–5 V range. After sample collection the $CO_2$ concentration of air contained within the vacutainers was determined using closed system respirometry (Vleck 1987). Briefly, vacutainer pressure was measured by inserting a needle attached to a Sable Systems PT-100 pressure meter through the vacutainer septa. A 1-ml sample was then withdrawn from the vacutainer using a precision 1-ml Gastight Hamilton syringe. This sample was introduced into a LiCor 6251 Carbon Dioxide analyzer by injection into a dry $CO_2$ free carrier gas flowing through the analyzer at 50 ml/min as regulated by a mass flow controller. Carbon dioxide concentration was determined by integrating the peak generated by each sample using Sable Systems DATA CAN V and corrected to standard conditions.

$\delta^{13}C$ values were plotted against the reciprocal of $CO_2$ concentration for each sequence of samples. The *y*-intercept of these Keeling plots was used as an estimate of the $\delta^{13}C$ of exhaled $CO_2$. We compared the $\delta^{13}C$ of fed and fasted birds using paired *t*-tests. We also compared the $\delta^{13}C$ of exhaled $CO_2$ with that of the birds' diets using one-sample *t*-tests.

## Field measurements

Samples for Keeling plots were also obtained from wild caught White-winged and Mourning Doves feeding on natural diets. Birds were captured with walk-in traps in July 1998 at the Sand Tank Mountains, Pima County, Ariz., USA. All birds were captured between 0600 and 0830 hours. Within 10 min of capture, birds were placed inside of 9-l Igloo coolers with a small 12-V electric squirrel-cage fan attached to the inside of the cooler's lid. The fan was powered by a 12-V car battery and served to mix the air within the chamber to prevent stratification of carbon dioxide. A 7-mm hole penetrating the lid allowed for the placement of a 7-mm BEV-Line sampling tube into the chamber. Samples were drawn from the chamber by attaching a 30-ml BD syringe to the BEV-line tubing and then withdrawing the plunger to the 20-ml mark. Birds were placed in the chamber and five sequential samples were obtained starting immediately after the bird's introduction and then at 1- to 2-min intervals. Carbon isotope ratios of samples were measured at the University of Utah's SIRFER isotope facility using a continuous flow Finnegan MAT 252 isotope ratio mass spectrometer with a pre-concentrating device. $CO_2$ concentration was measured as described above.

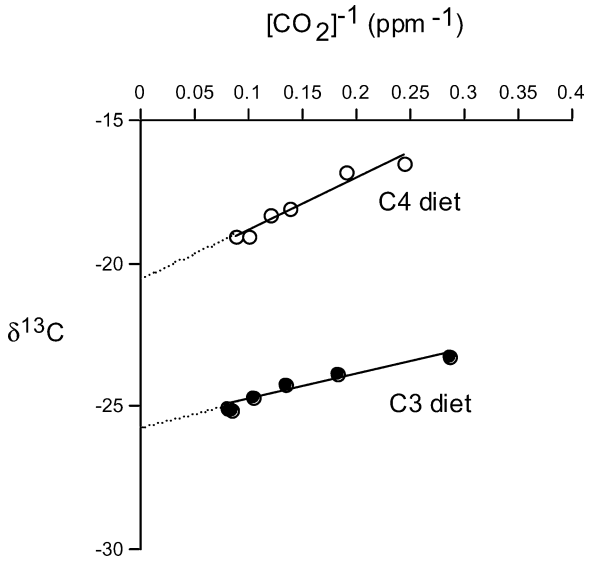


**Fig. 1** The isotopic composition of an animal's breath can be estimated from Keeling plots in which the $\delta^{13}C$ value of the $CO_2$ of air samples is plotted against the reciprocal of the $CO_2$ concentration in these samples. The two examples are for Rufous Hummingbirds (*Selasphorus rufus* fed on a C4 (*open symbols*, $y$=18.087$x$−20.626, $r^2$=0.95) and C3 (*closed symbols*, $y$=8.933$x$−25.664, $r^2$=0.93) diet. Air samples were drawn at 2, 4, 8, 12, 16, 20, and 24 min. The *y*-intercept of these regression lines estimates the $\delta^{13}C$ of respired $CO_2$

$\delta^{13}C$ values were plotted against the reciprocal of $CO_2$ concentration for each sequence of samples. The *y*-intercept of these Keeling plots was used as an estimate of the $\delta^{13}C$ of exhaled $CO_2$. In the case of wild-caught birds, we expected the $\delta^{13}C$ of exhaled $CO_2$ in White-winged Doves to reflect that of saguaro fruit ($\delta^{13}C$=−13.1 ±0.3‰, $n$=28), which is the primary food source in July at our study site (Wolf and Martinez del Rio 2000). We expected the $\delta^{13}C$ of exhaled $CO_2$ in Mourning Doves to reflect that of C3 seeds at our study site ($\delta^{13}C$=−24.9 ±0.7‰, $n$=6; Wolf et al. 2002).

## Results

In all cases there was a tight linear relationship between $\delta^{13}C$ and $[CO_2]^{-1}$ (average $r^2$±SD=0.83±0.12). Figure 1 shows two representative measurements for birds on the C3 and C4 diet. For birds fed on the C3 diet, the estimated $\delta^{13}C$ (mean ± SD) of respired $CO_2$ (−25.1±0.2‰ VPDB,

$n$=6, Fig. 2a) was not significantly different than that of their diet (−25.1±0.1‰ VPDB, $n$=5, $t$=0.4, $p$>0.1, Fig. 2a). The $\delta^{13}C$ of respired $CO_2$ of fasted birds (−25.7±0.4‰ VPDB, $n$=6, Fig. 2b) was slightly, albeit statistically significantly, more negative than that of their diet ($t$=8.5, $p$<0.001, Fig. 2b).

Before switching to a C4 diet, the $\delta^{13}C$ of respired $CO_2$ of fasted birds (−25.5±0.2 ‰ VPDB, $n$=6) was very similar to that of fasted birds in the C3/C3 experiment. After feeding on the C4 diet for 6 h, the $\delta^{13}C$ of respired $CO_2$ changed significantly to −19.2±2.5‰ VPDB (Fig. 2c, paired $t$=5.85, $p$<0.01, $n$=6). We were surprised to find that although this value was more positive than that of the C3 diet, it was also significantly more negative than that of the C4 diet ($t$=7.9, $p$<0.0001). This result indicates that the carbon in the bird's breath had two sources: ingested C4 sugar and endogenous reserves with a C3 composition.

The keeling plots obtained in the field were also well described by linear functions (average $r^2$±SD=0.95 ±0.03). The $\delta^{13}C$ of respired $CO_2$ estimated by Keeling plots for White-winged Doves (−15.9±0.8‰ VPDB, Fig. 3) was similar that of the $\delta^{13}C$ of Saguaro fruits (≈−13.1±0.3‰ VPDB; Wolf et al. 2002). The $\delta^{13}C$ of respired $CO_2$ for Mourning Doves (−23.5±1.5‰ VPDB, Fig. 3) was similar to that of the $\delta^{13}C$ of C3 seeds (−24.9±0.3‰ VPDB; Wolf et al. 2002). Because our sample sizes in the field were small, we did not use inferential statistics to compare between the $\delta^{13}C$ values of breath and those of the resources used by the doves.

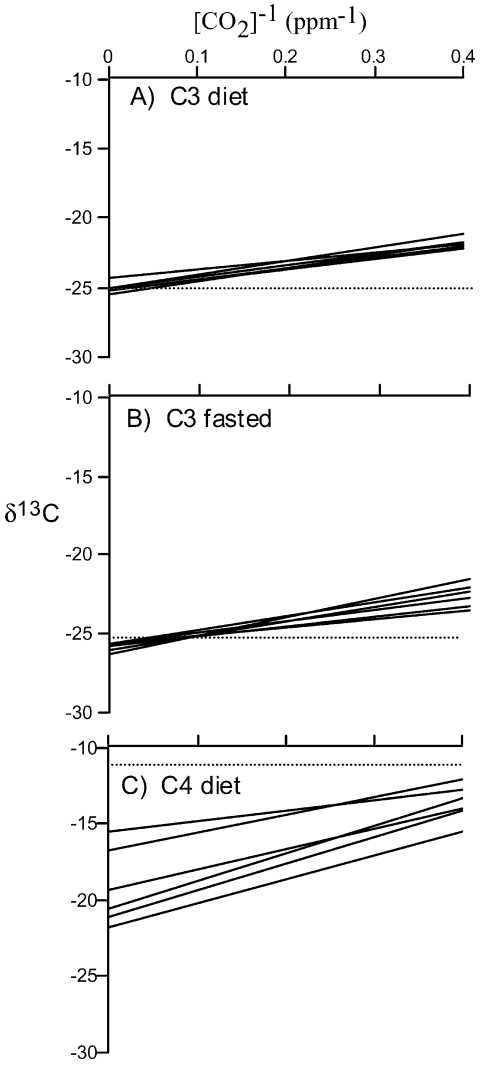


**Fig. 2** **a** When hummingbirds were fed for 3 months on a C3 diet, the $\delta^{13}C$ of their respired $CO_2$ was indistinguishable from that of their diet (*dashed line*). **b** When these birds were fasted overnight, the $\delta^{13}C$ of their respired $CO_2$ was slightly, albeit significantly, depleted in $^{13}C$ (*dashed line*). **c** When these birds were switched to a C4 diet for 6 h, the $\delta^{13}C$ of their respired $CO_2$ approached, but did not equal, that of their new diet (*dashed line*). *Each line* represents the regression for an individual

## Discussion

Our results suggest that Keeling plots can be used to measure the $\delta^{13}C$ of animals ranging in size from hummingbirds (≈3 g) to White-winged Doves (≈144 g) in either laboratory or field. The method is easy to implement and the price of the equipment needed to use it is small. Our results revealed broad concordance between an animal's diet and the isotopic composition of its breath both in the laboratory and in the field. They also yielded a surprise. We expected the $\delta^{13}C$ of hummingbird breath to be identical to that of the food that they were ingesting. Instead, we found that after 6 h of feeding the $\delta^{13}C$ of their breath was intermediate between that of their food and that of the food ingested before. Here we consider the advantages and disadvantages of Keeling plots to measure $\delta^{13}C$ in the breath of small animals. We also discuss whether $\delta^{13}C$ is a good "instantaneous" indicator of the isotopic composition of diet.

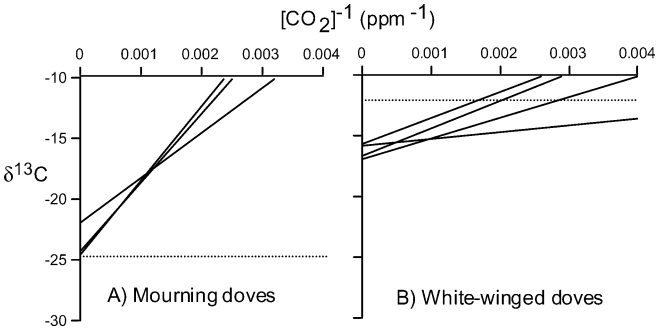


**Fig. 3a, b** Keeling plots for **a** Mourning (*Zenaida macroura*) and **b** White-winged (*Z. asiatica*) Doves showed that these animals respired $CO_2$ with a composition similar to that of their food plants: C3 seeds (−24.9±0.3‰ VPDB and saguaro cactus fruit (−13.1 ±0.3‰ VPDB)), respectively. *Dashed lines* represent the $\delta^{13}C$ of food sources for Mourning and White-winged Doves (Wolf and Martinez del Rio 2002). *Each line* represents a Keeling plot for an individual bird

## Advantages and disadvantages of Keeling plots

Our initial interest in this method was its potential application for field studies. The two chambers (Tupperware and Igloo coolers) used for the collection of gas samples are very inexpensive. In addition, setting up of the sampling equipment is simple. While choosing a chamber, it is important to consider the size of the animal in relation to the volume of the chamber. The chamber must be small enough for respired $CO_2$ to accumulate to high enough levels in a reasonable amount of time. In our hummingbird experiments, the minimum amount of time needed for $CO_2$ to accumulate to levels that allowed estimating $\delta^{13}C$ in 100 to 300-μl air samples was 4 min. The method does not require transportation of animals to a central handling location and nor does it require large and often awkward $O_2$ and $CO_2$ gas tanks, or species-specific gas masks (Hatch et al. 2002a). It is especially useful for field studies in which researchers expect to use it on a variety of animals. Animals can be handled, sampled and released at the site of capture in less than 15 min. Its major disadvantage is cost. Whereas a previously published method requires analyzing the isotopic composition of only one sample (Hatch et al. 2000a, b), the method described here requires from four to six samples to a minimum of three samples per animal. In all methods that use regression to estimate the value of a parameter, more points imply more accurate estimation and a smaller confidence interval for the estimate (Zar 1996). Keeling plots are no exception. Our recommendation is to use as many samples as is cost effective to at least three samples, with four to six samples providing very high confidence. Compared to the method described in Hatch et al. (2000a, b), the cost of sample analyses is increased and thus the flexibility and ease of the method comes at a cost.

## Does $\delta^{13}C$ in breath reflect the isotopic composition of current diet?

Many authors have assumed that the $\delta^{13}C$ of respired $CO_2$ estimates the $\delta^{13}C$ of the substrate being metabolized (Schoeller et al. 1980, 1984; Normand et al. 1992). Hatch et al. (2002b) found that in Rock Doves (*Columba livia*) the $\delta^{13}C$ of respired $CO_2$ did not begin to reflect the current diet until 20 h after a diet switch. When the doves were fasted, the $\delta^{13}C$ of respired $CO_2$ reverted back to that of the dove's long-term diet within 24 h.

Fasting Rufous Hummingbirds respired $CO_2$ with a $\delta^{13}C$ that was more negative than that of their long-term diet. This result is consistent with the observation that storage lipids synthesized from the carbon skeletons of carbohydrates tend to be depleted in $\delta^{13}C$ relative to their carbohydrate source (DeNiro and Epstein 1977). However, the $\delta^{13}C$ depletion in the $CO_2$ of fasted birds was small (≈0.6 ‰). The most commonly cited value for the difference in $\delta^{13}C$ between synthesized lipids and the carbohydrates that they are synthesized from is −3‰ (DeNiro and Epstein 1977). Two non-exclusive hypotheses can explain this discrepancy: (1) fasting hummingbirds catabolized a mixture of lipids and carbohydrates, and (2) the lipids of hummingbirds are not strongly depleted. Stott et al. (1997) have documented variation in the isotopic discrimination between diet and tissue fatty acids ($\Delta 13C_{\text{tissue fatty acids-diet}}$) ranging from −0.5 to −5‰. The isotopic composition of lipids catabolized may depend on the fatty acid composition of lipid deposits, which is unknown in hummingbirds.

When birds fed on a C3 diet for 3 months were fed for 6 h on a C4 diet, the $\delta^{13}C$ of their respired $CO_2$ reflected a mixture of C3 and C4 sources. We were surprised by this result and decided to apply a simple mixing model to estimate the relative contribution of these two diets to the substrates catabolized by hummingbirds. This model estimates the fraction contributed by the C4 diet (P) to the total $CO_2$ exhaled from the $\delta^{13}C$ values of this diet ($\delta^{13}C_{C4diet}$), the composition of fasted birds that had fed on the C3 diet ($\delta^{13}C_{C3fasted}$), and the composition of birds that had fed on the C4 diet ($\delta^{13}C_{C4fed}$; Dawson et al. 2002):

$$P = \frac{\delta^{13}C_{C4Fed} - \delta^{13}C_{C3Fasted}}{\delta^{13}C_{C4diet} - \delta^{13}C_{C3Fasted}} \quad (3)$$

The contribution of the C4 diet to catabolism ranged from 21 to 69% (mean ± SD=43.8±17.6). Animals as small as hummingbirds may catabolize a mixture of stored and recently assimilated nutrients. Thus, the $\delta^{13}C$ of breath does not always provide an instantaneous snapshot of the carbon isotope composition of an animal's current diet. It estimates the $\delta^{13}C$ of the substrates catabolized, which may or may not be what the animal ingested in the short time prior to measurement. To interpret the $\delta^{13}C$ values of respired $CO_2$, we must do experiments that assess the time course of the incorporation of a new diet into the $\delta^{13}C$ of an animal's respired $CO_2$. We suspect that the $\delta^{13}C$ of respired $CO_2$ will often reflect the composition of a diet ingested before measurement within a relatively short window. However, the length of this window will depend on a variety of factors such as the animal's size and its metabolic rate. As is the case with other tissues, we are just beginning to understand the determinants of the rate at which the isotopic composition of diet is incorporated into an animal's tissues and into the pool of nutrients that it uses as energy sources.

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