

Oxidation of dietary sugar during hovering flight in small vertebrate nectarivores

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10.1242/jeb.063792

There was an error published in Eqn 3 (p. 2156) of *J. Exp. Biol.* **210**, 2154-2162 and in Eqn 2 (p. 311) of *J. Exp. Biol.* **211**, 310-316.

In both papers, the following equation was written incorrectly as:

$$\delta^{13}\text{C}_{\text{breath}} = [\delta^{13}\text{C}_{\text{ambient}}(f_a) + \delta^{13}\text{C}_{\text{sample}}] / 1 - f_a$$

The equation should have read:

$$\delta^{13}\text{C}_{\text{breath}} = [\delta^{13}\text{C}_{\text{sample}} - \delta^{13}\text{C}_{\text{ambient}}(f_a)] / 1 - f_a$$

The authors apologise for any inconvenience this may have caused but assure readers that the error was typographical and does not affect the data, results, interpretations or conclusions of either paper.

Oxidation rate and turnover of ingested sugar in hovering Anna's (*Calypte anna*) and rufous (*Selasphorus rufus*) hummingbirds

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Accepted 2 May 2007

Summary

Hummingbirds obtain most of their dietary calories from floral nectar ingested during hovering flight. Despite the importance of dietary sugar to hummingbird metabolism, the turnover of newly ingested carbon in the pool of actively metabolized substrates has not been adequately characterized in hovering hummingbirds. By combining respirometry with stable carbon isotope analysis of respired breath, we show that in rufous (*Selasphorus rufus*) and Anna's (*Calypte anna*) hummingbirds at high foraging frequencies, utilization of newly ingested sugars increased over a period of 30–45 min until it accounted for virtually 100% of the fuel oxidized. This newly ingested sugar disappears from the actively metabolized pool of substrates over a similar time course. These results demonstrate that turnover of carbon in the pool of actively metabolized substrates is rapid; carbon from ingested sucrose is available for oxidation for

30–45 min before being cleared. By monitoring expired CO₂ for the appearance and disappearance of the signature characteristic of newly ingested sugar and then calculating energy budgets using video recordings of hummingbird activity, we estimated the proportion of recently ingested sugar used to fuel ongoing metabolism as well as the proportion devoted to energy storage. Consistent differences between species in the percentage of ingested cane sugar oxidized during the 2 h experimental periods suggest that individuals of each species adopted energy intake patterns appropriate to their needs. This approach provides a means by which to examine the partitioning of dietary carbon intake between energy expenditure and storage using non-invasive, field-compatible techniques.

Key words: energetics, hummingbird, stable isotope, turnover.

Introduction

Hummingbirds have served as exceptional model organisms for the study of foraging energetics due to their high visibility and cooperative nature in the wild, ease of use in the laboratory, high rates of energy intake and expenditure, and because most of the calories they ingest come from simple sugars in floral nectar. As hovering hummingbirds transition from a fasted to a fed state, they rapidly switch from oxidizing fatty acids to oxidizing carbohydrates (Suarez et al., 1990; Welch et al., 2006). Using broad-tailed hummingbirds *Selasphorus platycercus*, Welch et al. (Welch et al., 2006) found that recently ingested sugars can provide virtually all the fuel required for energy metabolism during repeated bouts of foraging. An important question concerns the rate of turnover of recently ingested sugars in the pool of actively metabolized substrates under such ecologically relevant circumstances. It is possible that ingested sugars remain as part of a pool of actively metabolized substrates for an extended period and that more recently ingested sugar molecules simply mix with this pool. Alternatively, ingested sugars may remain in the pool of

actively metabolized substrates for a brief period of time, being rapidly replaced by newly ingested sugar molecules as foraging proceeds. Carleton et al. (Carleton et al., 2006) previously estimated the turnover rate of carbon in endogenous stores of energy (fat) over a period of several days, but this work was not designed to examine carbon turnover at the shorter time scales relevant to birds engaged in repeated foraging bouts. The study presented here expands on these previous studies and quantifies the turnover rate of ingested sugar in the pool of actively metabolized substrates in actively foraging rufous (*Selasphorus rufus*) and Anna's (*Calypte anna*) hummingbirds.

Recent work with broad-tailed hummingbirds *S. platycercus* (Welch et al., 2006) has shown the feasibility of determining the contribution of ingested fuel to the fueling of oxidative metabolism during hovering flight. The methodology employed in this and previous studies (Carleton et al., 2006; Carleton et al., 2004; Welch et al., 2006) takes advantage of the difference in stable isotopic signature of carbon in sugar derived from two distinct plant sources. Sugar from sugar beets displays a carbon stable isotope ratio particular to plants with

a C3 photosynthetic pathway, while sugar from sugar cane displays a distinct carbon stable isotope ratio particular to plants with a C4 photosynthetic pathway. By varying the availability of sugar from each source and then tracking the isotopic composition of the CO₂ expired by the birds, it is possible to determine the contribution of dietary sugar to oxidative metabolism. These studies benefit from the ability to conduct repeated measurements over time, and may be conducted under natural field conditions. Because hummingbirds absorb nearly all of the sugar they ingest (Karasov et al., 1986; McWhorter et al., 2006), sugar not oxidized is reserved for energy storage by conversion to glycogen or fat. Thus, by monitoring sugar intake, and by tracking the use of ingested sugar *via* stable isotope analysis of expired CO₂, it is possible to estimate rates of net energy storage non-invasively using the same individual.

Thus, our goals in the studies reported here were: first, to determine the timing and extent to which Anna's and rufous hummingbirds support hovering flight with newly ingested sugars. We hypothesize that the ability to rely primarily on recently ingested sugar to fuel oxidative metabolism during flight is a trait common to hummingbirds. If so, then Anna's and rufous hummingbirds should oxidize recently ingested sugars as quickly and extensively as broad-tailed hummingbirds (Welch et al., 2006). Second, we wished to determine the rate of turnover of newly ingested sugar within the pool of actively metabolized substrates, while mimicking conditions experienced by foraging wild hummingbirds. We hypothesize that recently ingested sugars are removed from the pool of actively metabolized substrates approximately as quickly as they are incorporated. Third, we wished to evaluate the rate of net energy gain by Anna's and rufous hummingbirds by combining stable isotope tracking of carbon in expired CO₂ with monitoring of nectar intake and energy expenditure.

Materials and methods

We report $\delta^{13}\text{C}$ on a per mil (‰) basis relative to the international carbon standard, Vienna Pee Dee Belemnite (VPDB), where:

$$\delta^{13}\text{C} = \frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}} - (^{13}\text{C}/^{12}\text{C})_{\text{standard}}}{(^{13}\text{C}/^{12}\text{C})_{\text{standard}}} \times 10^3. \quad (1)$$

All solid and gas samples were submitted to the University of California, Santa Barbara Marine Science Institute Analytical Laboratory for analysis of $^{13}\text{C}/^{12}\text{C}$ ratios by mass spectrometry.

This facility utilizes a Roboprep-CN stable isotope ratio mass spectrometer (Europa Scientific, Crew, UK) equipped with an autosampler for introduction of gas samples into the continuous flow combustion chamber.

Animal care and experimental design

All hummingbirds were captured with a modified Hall trap (Russell and Russell, 2001). Individual *Selasphorus rufus* Gmelin 1788 (body mass at start of experiment=3.4±0.2 g; N=4, 2 male/2 female) were captured in Inyo, Mono and Santa Barbara Counties in California, USA. Individual *Calypte anna* Lesson 1829 (body mass at start of experiment=4.8±0.8 g; N=3, 2 male/1 female) were captured in Santa Barbara County, California, USA. Captive hummingbirds were housed at the UCSB Aviary in individual outdoor, wire-mesh enclosures measuring 1.8 m tall by 0.6 m wide by 2.4 m long. Birds were fed *ad libitum* on a 13% (w/v) solution of Nektar-Plus (Guenter Enderle, Tarpon Springs, FL, USA) supplemented with beet sugar (5% w/v). The $\delta^{13}\text{C}$ value of the maintenance diet was -25.84±0.11‰ (N=10; Table 1). Birds were subjected to natural photophase and ambient weather conditions. Capture, housing and experimental protocols were approved by the University of California, Santa Barbara Institutional Animal Care and Use Committee (Protocol 672).

Data collection was conducted in an enclosure measuring 0.92 m wide×0.54 m high×0.51 m deep, in the laboratory at an average temperature of 24.0±0.3°C. The only perch available to the hummingbird within the cage was placed on top of a balance, which was monitored in order to determine bird mass. Data collection took place between August and October of 2006 between 06:00 h and 11:00 h. Prior to each experiment, the selected hummingbird was fasted overnight to ensure that it would be oxidizing primarily fat at the beginning of the period of data collection (Suarez et al., 1990; Welch et al., 2006).

Experimental design and data collection were based largely on those reported in Welch et al. (Welch et al., 2006). Following the overnight fast of approximately 8–10 h, hummingbirds were provided with a disposable 20 ml syringe containing a solution of cane (C4 photosynthetic pathway) sugar (20% w/v). The $\delta^{13}\text{C}$ value of this solution was -11.63±0.11‰ (N=10; Table 1) and it was available to the bird for approximately 1 h. The syringe containing the cane sugar solution was weighed to the nearest 0.0001 g immediately prior to and following the period that it was available to the bird. The difference in mass before and after the period it was available

Table 1. Stable carbon isotope signatures ($\delta^{13}\text{C}$) of maintenance diet and sucrose solutions utilized during experimental protocol

Solution (% w/v)	$\delta^{13}\text{C}$ (‰, VPDB)	N
13% Nektar Plus + 5% beet sugar (maintenance diet)	-25.84±0.11	10
20% C4 sucrose solution (First hour)	-11.63±0.11	10
20% C3 sucrose solution (Second hour)	-24.02±0.11	10

Values are means ± s.d.

VPDB, Vienna Pee Dee Belemnite C standard.

to the bird was taken as the mass of the solution ingested. As the density of solid sucrose is 1.587 g cm^{-3} , a 20% w/v sucrose solution is equal to an 18.6% w/w solution. Given that the specific density of an 18.6% w/w sucrose solution is 1.07677 (Horwitz, 1975), this means that the mass of sucrose ingested ($\text{Suc}_{\text{ingest}}$; in g) may be determined by the following equation:

$$\text{Suc}_{\text{ingest}} = (\text{Sol}_{\text{ingest}} / \rho_{\text{sol}}) \times 0.186, \quad (2)$$

where $\text{Sol}_{\text{ingest}}$ is the mass of cane sugar solution ingested by the bird (in g), ρ_{sol} is the specific gravity of an 18.6% w/w sucrose solution and 0.186 is the proportion (w/w) of the solution that is sucrose.

Immediately after removing and weighing the cane sugar solution, the hummingbird was provided with a 20% w/v beet sucrose solution. The $\delta^{13}\text{C}$ value of the beet sucrose solution was $-24.02 \pm 0.11\text{‰}$ ($N=10$; Table 1). This solution was available to the hummingbird for approximately one additional hour, such that the total time allotted to this experiment for each individual was 2 h.

Respirometry

Hummingbirds had to hover to feed, inserting their head into a plastic tube extending from the front of the feeder. This tube was derived from a disposable 30 ml syringe and, except for the front opening, was airtight. Halfway along its length, plastic tubing was attached to the mask, allowing incurrent air to be drawn through the mask and delivered to respirometry equipment. Air first passed through a column of DrieriteTM (W. A. Hammond Drierite, Xenia, OH, USA) to scrub water vapor before entering the carbon dioxide analyzer (CA-2A, Sable Systems International, Las Vegas, NV, USA). After leaving the carbon dioxide analyzer, air passed through a DrieriteTM-AscariteTM-DrieriteTM column (Ascarite II, Arthur H. Thomas, Philadelphia, PA, USA), to scrub any carbon dioxide and additional water from the line, and then into the oxygen analyzer (FOXBOX, Sable Systems International). Air flow was maintained by a mechanism internal to the FOXBOX (thus, after the removal of water vapor) at a rate of 500 ml min^{-1} . An infrared emitter and receiver were placed on either side of the front edge of the mask such that the infrared beam was disrupted by the presence of the hummingbird's head in the mask. By determining the length of time the infrared emitter was occluded, we were able to resolve the duration of any feeding event (and subsequent gas analysis event). The signal from the infrared receiver, along with data from the carbon dioxide analyzer, oxygen analyzer and balance, was reported to a notebook computer for recording *via* connection to a Universal Interface II (Sable Systems International). Data were recorded at 0.05 s intervals for 2 h using Expedata software (v. 1.0.17, Sable Systems International).

Immediately before data collection, the oxygen analyzer was calibrated with well-mixed ambient air drawn through the mask in the absence of a hummingbird. The carbon dioxide analyzer was calibrated with CO_2 -free nitrogen gas (zero gas) and 0.5% CO_2 in nitrogen gas (Praxair, Danbury, CT, USA). In each case,

tubing was removed directly downstream of the mask and held inside a small reservoir into which flowed the calibration gas at a rate in excess of the flow rate of air pulled through the respirometry system.

STP-corrected oxygen depletion and carbon dioxide enrichment associated with each feeding event were determined after first correcting by subtracting baseline values (determined as the linear extrapolation of points directly before and after the feeding event in question). These baseline-corrected data were then converted to ml of gas by application of standard equations (Withers, 1977). Determination of absolute rates of oxygen consumption and carbon dioxide production was not possible during this experiment because subsampling of incurrent air was attempted in each case (see below). However, as subsampling did not discriminate between oxygen and carbon dioxide, relative volumes (ml) of oxygen and carbon dioxide respired by the bird were determined. These were obtained by integrating the gas depletion or enrichment peak over time (min) and used to calculate respiratory quotient (RQ).

For the purpose of estimating metabolic rate of hovering hummingbirds during this experiment, complementary measurements of \dot{V}_{O_2} and \dot{V}_{CO_2} during hover-feeding were obtained for all individuals. These measurements were taken on the same day approximately 2 h after the experiment described above. Flow rate was held at 1200 ml min^{-1} and no expired breath subsamples were taken (see below). Otherwise, the methodology adopted during this complementary data collection period was identical to that described above.

Collection and analysis of expired CO_2

Expired CO_2 was collected while hummingbirds were hover-feeding at the respirometry mask by drawing air from the incurrent airline approximately halfway between the mask and the carbon dioxide analyzer *via* a 60 ml syringe (Welch et al., 2006). These samples contained both ambient CO_2 as well as CO_2 expired by the hummingbird. Thus, in order to estimate $\delta^{13}\text{C}$ of respired breath ($\delta^{13}\text{C}_{\text{breath}}$) we used a two-part concentration-dependent mixing model adapted from Phillips and Koch (Phillips and Koch, 2002), such that:

$$\delta^{13}\text{C}_{\text{breath}} = [\delta^{13}\text{C}_{\text{ambient}}(f_a) + \delta^{13}\text{C}_{\text{sample}}] / 1 - f_a, \quad (3)$$

where $\delta^{13}\text{C}_{\text{sample}}$ is $\delta^{13}\text{C}$ of air collected in the syringe. $\delta^{13}\text{C}_{\text{ambient}}$ is average $\delta^{13}\text{C}$ of air collected at three points during the 2-h experimental period (one within the first 15 min, one near the halfway point, and one within 20 min of the end of the 2 h period) in the same manner as above when a hummingbird was not present at the mask. f_a is the fraction of CO_2 in the gas sample from ambient air. Ambient $[\text{CO}_2]$ (p.p.m.) was determined using the carbon dioxide analyzer immediately before a feeding bout. $[\text{CO}_2]$ (p.p.m.) of the air sample was determined during stable isotope analysis by the University of California, Santa Barbara Marine Science Analytical Lab. Immediately following collection, contents of the 60-ml syringe were injected into pre-evacuated 12 ml Exetainer vials (Labco Limited, Buckinghamshire, UK) until a positive

pressure was achieved. Samples were stored at room temperature for as long as 5 days before submission for analysis. All data associated with individual feeding events having $\delta^{13}\text{C}_{\text{breath}}$ values for which the CO_2 concentration of the sample was not at least twice the CO_2 concentration of the ambient air were excluded from further analysis.

Time and energy budgets

The activity of hummingbirds was recorded on videotape during the entirety of the 2-h experimental period. The recording period was divided into 2 min blocks for further analysis, with the first block beginning when the hummingbird first fed from the suspended feeder. Hummingbird activity was classified as either hovering/flying or perching. The proportion of each 2 min block devoted to either activity was quantified.

Energy expenditure (in ml O_2) during each 2 min block was determined by multiplying the amount of time spent either hovering/flying or perching by metabolic rates associated with each activity. As described above, hovering metabolic rate (in $\text{ml O}_2 \text{g}^{-1} \text{h}^{-1}$) was estimated *via* measurement of mass-specific oxygen consumption rate for each hummingbird during complimentary experiments. The relatively small size of the experimental enclosure greatly constrained the forward flight speed of the hummingbirds. Estimates of the oxygen consumption rate in small hummingbirds as a function of flight speed indicate a relatively flat relationship at low flight speeds, suggesting metabolic rate during hovering is equal to metabolic rate during forward flight in this range (Berger and Hart, 1972). As a result, we assume the metabolic cost of low-speed forward flight within the enclosure to be equal to the cost of hovering. We ignore the energetic costs of acceleration and deceleration, as good estimates of these do not exist. Estimates of mass-specific oxygen consumption rate (in $\text{ml O}_2 \text{g}^{-1} \text{h}^{-1}$) during perching for both *C. anna* and *S. rufus* were taken from Lasiewski's seminal work (Lasiewski, 1963). These mass-specific oxygen consumption rates were multiplied by an estimate of the hummingbirds mass (as described above) during the feeding event closest in time to each 2 min period to obtain total metabolic rates (MR_{block} ; in $\text{ml O}_2 \text{h}^{-1}$) for each activity for that period.

As described above, estimates of the respiratory quotient were obtained for each feeding event. Assuming hummingbirds oxidize primarily fat and/or carbohydrate (Suarez et al., 1990; Welch et al., 2006), total energy expenditure during each 2 min period (E_{block} ; in J) can be estimated as:

$$E_{\text{block}} = \{[(1-\text{RQ})/0.29][h_{\text{O}_2(\text{fat})}] + [(1-\text{RQ})/0.29][h_{\text{O}_2(\text{carb})}]\} \times \{t_{\text{hov}}[\text{MR}_{\text{block}(\text{hov})}/3600] + t_{\text{perch}}[\text{MR}_{\text{block}(\text{perch})}/3600]\}, \quad (4)$$

where RQ is the respiratory quotient for the feeding event closest to that 2 min block (constrained to be between 0.71 and 1.0), $h_{\text{O}_2(\text{fat})}$ is the thermal equivalent of oxygen exchange when fat is the metabolic substrate (19.8 J ml^{-1}) (Brouwer, 1957), $h_{\text{O}_2(\text{carb})}$ is the thermal equivalent of oxygen exchange when carbohydrates are the metabolic substrate (21.1 J ml^{-1}) (Brouwer, 1957), t_{hov} and t_{perch} are the duration of time spent

hovering/flying and perching (in s), respectively, during that 2 min block, and $\text{MR}_{\text{block}(\text{hov})}$ and $\text{MR}_{\text{block}(\text{perch})}$ are the rates of oxygen consumption (in $\text{ml O}_2 \text{h}^{-1}$) for hovering/flying and perching, respectively, during that 2 min block.

Determination of cane sugar oxidation rate

A non-linear function was fitted to $\delta^{13}\text{C}_{\text{breath}}$ values during the first hour (feeding events for which cane sugar solution was available) and separately to $\delta^{13}\text{C}_{\text{breath}}$ values during the second hour (feeding events for which beet sugar solution was available). Thus, instantaneous estimates of $\delta^{13}\text{C}_{\text{breath}}$ values were possible. We assume that the incorporation of carbon into expired CO_2 can be approximated by single-compartment, first-order kinetics (Carleton et al., 2006). The non-linear fitting formula is:

$$\delta^{13}\text{C}_{\text{breath}}(t) = \delta^{13}\text{C}_{\text{breath}}(\infty) + [\delta^{13}\text{C}_{\text{breath}}(0) - \delta^{13}\text{C}_{\text{breath}}(\infty)]e^{-kt}, \quad (5)$$

where $\delta^{13}\text{C}(t)$ is the isotope composition of the carbon in expired CO_2 at time t , $\delta^{13}\text{C}_{\text{breath}}(0)$ is the estimated initial isotope composition of the carbon in expired CO_2 , $\delta^{13}\text{C}_{\text{breath}}(\infty)$ is the asymptotic equilibrium isotope composition of the carbon in expired CO_2 and k is the fractional rate of isotope incorporation into the pool of expired CO_2 (Carleton et al., 2006; Carleton and Martínez del Rio, 2005; O'Brien et al., 2000). The subscript 'i' (for incorporation) will be used to indicate the application of each of these variables to the period of the experiment during which cane sugar solution is available. The subscript 'd' (for disappearance) will be used to indicate the application of each of these variables to the period of the experiment during which beet sugar solution is available. For each 2 min block for which time budgets were estimated an average $\delta^{13}\text{C}_{\text{breath}}$ value was estimated by solving Eqn 5 with time (t) equal to the median value for that 2 min block (in min).

This $\delta^{13}\text{C}_{\text{breath}}$ value provides a means of estimating the proportion of expired CO_2 derived from oxidation of exogenous carbohydrate (Carleton et al., 2006; Welch et al., 2006). Specifically, the fraction of expired CO_2 derived from oxidation of cane sugar (f_{exo}) during any 2 min block was estimated as:

$$f_{\text{exo}} = (\delta^{13}\text{C}_{\text{breath}} - \delta^{13}\text{C}_{\text{C}_3}) / (\delta^{13}\text{C}_{\text{C}_4} - \delta^{13}\text{C}_{\text{C}_3}), \quad (6)$$

where $\delta^{13}\text{C}_{\text{C}_4}$ is the $\delta^{13}\text{C}$ value of the cane sugar solution and $\delta^{13}\text{C}_{\text{C}_3}$ is the $\delta^{13}\text{C}$ value of endogenous fuels [estimated as $\delta^{13}\text{C}_{\text{breath}}(0)$ from Eqn 6], during the first hour of the experiment, and $\delta^{13}\text{C}_{\text{C}_3}$ is the $\delta^{13}\text{C}$ value of the beet sugar solution during the second hour of the experiment.

For each mol sucrose oxidized, 12 mol O_2 are consumed ($2 \times 6 \text{ mol O}_2$ per unit hexose). Thus, the amount of cane sugar oxidised (M_{cane} ; in μmol) during each 2 min period may be estimated as:

$$M_{\text{cane}} = [f_{\text{exo}}(\text{Met}_{\text{block}}) \times 10^6] / 12, \quad (7)$$

where $\text{Met}_{\text{block}}$ is the amount of O_2 (in mol) consumed during that 2 min block.

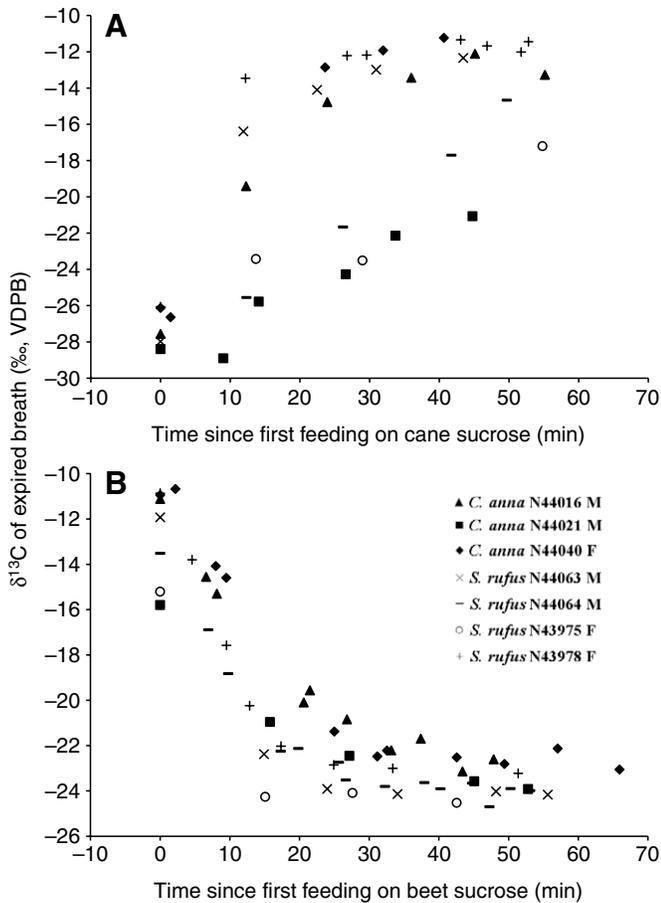


Fig. 1. The stable carbon isotopic signature of expired CO_2 ($\delta^{13}\text{C}_{\text{breath}}$ in ‰, VPDB) in relation to (A) the time since the first feeding on cane sucrose solution (C4) and the consequent (B) time since the first feeding on beet sucrose solution (C3). M, male; F, female.

Results

RQ values during the first feeding event following a fast were near 0.71 in both rufous and Anna's hummingbirds (*S. rufus*: 0.74 ± 0.01 , $N=2$; *C. anna*: 0.76 ± 0.02 , $N=3$), indicating that the birds relied primarily on the oxidation of fatty acids to fuel hovering flight. After 40 min of access to food, average RQ values for each bird were approximately 1.0 and remained near this value for the remainder of the experiment in both species (*S. rufus*: 1.01 ± 0.01 , $N=4$; *C. anna*: 1.01 ± 0.01 , $N=3$), indicating that the birds had switched to fuelling hovering flight exclusively with carbohydrates.

During the first feeding bout following the fast, $\delta^{13}\text{C}_{\text{breath}}$ values were near the $\delta^{13}\text{C}$ value of the maintenance diet containing beet sugar and increased over the first hour towards the $\delta^{13}\text{C}$ value of cane sugar in the experimental diet (Fig. 1A, Table 2). $\delta^{13}\text{C}_{\text{breath}}$ averaged $-27.02 \pm 1.36\text{‰}$ ($N=2$) in *S. rufus*, and $-27.36 \pm 1.15\text{‰}$ ($N=3$) in *C. anna* during the first feeding event. For both species, this value was more negative than, but not significantly different from, the $\delta^{13}\text{C}$ value of the maintenance diet (*S. rufus*: $t_1 = -1.2299$, $P=0.4346$; *C. anna*: $t_2 = -2.2889$, $P=0.1493$). The fractional rate of isotopic

incorporation into the pool of expired CO_2 (k_i) varied extensively between individuals. k_i averaged $7.1 \pm 7.6\%$ (min^{-1} ; range 0.7–16.2%; $N=4$) in *S. rufus*. k_i averaged $4.5 \pm 3.7\%$ (min^{-1} ; range 0.2–7.1%; $N=3$) in *C. anna*. During a period of availability of cane sugar solution, when $\delta^{13}\text{C}_{\text{breath}}$ values had reached a plateau (the period beginning 40 min after the first feeding on cane sucrose), the proportion of metabolism (i.e. \dot{V}_{CO_2}) fuelled by dietary cane sugar (f_{exo}) approached 100%, similar to results shown previously in *S. platycercus* (Welch et al., 2006). f_{exo} averaged 0.83 ± 0.18 (range 0.61–1.01; $N=4$) for *S. rufus* during this steady state period of feeding on cane sugar (Fig. 1A, Table 2). f_{exo} averaged 0.81 ± 0.31 (range 0.46–1.03; $N=3$) for *C. anna* during this steady state period of feeding on cane sucrose (Fig. 1A, Table 2).

When the diet was switched from cane sugar back to beet sugar (the second hour of the experiment), the decrease in $\delta^{13}\text{C}_{\text{breath}}$ over time mirrored the increase in $\delta^{13}\text{C}_{\text{breath}}$ seen during the previous period of cane sugar feeding. There was less variability between individuals in the fractional rate of disappearance of labelled carbon in expired CO_2 compared to ^{13}C enrichment curves observed during the previous hour (Fig. 1B). The fractional rate of isotopic disappearance from the pool of expired CO_2 (k_d) averaged $10.9 \pm 2.9\%$ (min^{-1} ; range 9.2–13.5; $N=4$) in *S. rufus*. k_d averaged $5.7 \pm 0.4\%$ (min^{-1} ; range 5.3–5.9; $N=3$) in *C. anna*. During the period of beet sucrose availability when $\delta^{13}\text{C}_{\text{breath}}$ values had reached a plateau (the period beginning at least 40 min after the first feeding on beet sucrose) $\delta^{13}\text{C}_{\text{breath}}$ values neared the $\delta^{13}\text{C}$ signature of the beet sucrose solution. $\delta^{13}\text{C}_{\text{breath}}$ averaged $-23.97 \pm 0.54\text{‰}$ ($N=4$) for *S. rufus* and $-23.08 \pm 0.59\text{‰}$ ($N=3$) for *C. anna*. These values are not significantly different from the $\delta^{13}\text{C}$ signature of the beet sucrose solution (*S. rufus*: $t_3 = 0.1955$, $P=0.8575$; *C. anna*: $t_2 = 2.7620$, $P=0.1099$).

RQ and $\delta^{13}\text{C}_{\text{breath}}$ values were highly significantly correlated during the period of cane sugar availability in both rufous and Anna's hummingbirds (data pooled by species; Fig. 2; *S. rufus*: $r_{20} = 0.9494$, $P < 0.0001$; *C. anna*: $r_{17} = 0.9065$; $P < 0.0001$), suggesting that newly ingested sugars were fuelling metabolism during this period. By contrast, there was no significant correlation between RQ and $\delta^{13}\text{C}_{\text{breath}}$ values for either species during the period of beet sugar availability (data pooled by species; *S. rufus*: $r_{36} = -0.1880$, $P=0.2722$; *C. anna*: $r_{29} = 0.3294$, $P=0.0810$). As RQ remained near 1.0 during the entire period of beet sugar availability, no correlation would be expected.

Hummingbirds engaged in flight for varying amounts of time over the approximately 2-h period of video recordings beginning with their first hover-feeding event (Fig. 3, Table 3). *S. rufus* spent an average of $6.6 \pm 3.4\%$ (range: 3.0–9.6%; $N=4$) of the time hovering or flying. *C. anna* spent an average of $9.6 \pm 7.4\%$ (range: 5.3–18.1%; $N=3$) of the time hovering or flying. Consequently, hummingbirds expended varying amounts of energy during the approximately 2-h long period (Table 3). *S. rufus* expended an average of 1551 ± 259 J (range: 1202–1820 J; $N=4$), while *C. anna* expended an average of 2132 ± 748 J (range: 1297–2737 J; $N=3$).

Table 2. Kinetics of change in $\delta^{13}\text{C}_{\text{breath}}$ values over the course of the experiment for rufous (*S. rufus*) and Anna's (*C. anna*) hummingbirds

Bird	ID	Sex	k_i (min^{-1})	k_d (min^{-1})	f_{exo}
<i>S. rufus</i>					
	N43975	F	0.007	0.135	0.61
	N43978	F	0.162	0.092	1.01
	N44063	M	0.105	0.132	0.96
	N44064	M	0.009	0.077	0.75
	Mean \pm s.d.		0.071 \pm 0.076	0.109 \pm 0.029	0.83 \pm 0.18
<i>C. anna</i>					
	N44016	M	0.061	0.053	0.94
	N44021	M	0.002	0.059	0.46
	N44040	F	0.071	0.059	1.03
	Mean \pm s.d.		0.045 \pm 0.037	0.057 \pm 0.004	0.81 \pm 0.31

k_i , fractional rate of isotope incorporation into pool of expired CO_2 ; k_d , fractional rate of isotope disappearance from pool of expired CO_2 ; f_{exo} , proportion of expired CO_2 derived from oxidation of cane sugar [during period of availability of cane sucrose solution when $\delta^{13}\text{C}_{\text{breath}}$ values had reached a plateau (defined as period beginning 40 min after first feeding on cane sucrose)].

Hummingbirds also ingested variable total amounts of cane sugar solution (Table 3). *S. rufus* ingested an average of 0.601 ± 0.224 ml ($N=4$) of cane sugar solution, equivalent to 350.9 ± 131.0 μmol ($N=4$) of sucrose. *C. anna* ingested an average of 0.347 ± 0.278 ml ($N=3$) of cane sugar solution, equal to 352.2 ± 142.6 μmol ($N=3$) of sucrose.

The amount of ingested cane sugar oxidized by individual hummingbirds over the 2-h experimental period varied widely (Table 3). *S. rufus* oxidized an average of 109.5 ± 34.3 μmol ($N=4$) while *C. anna* oxidized an average of 160.5 ± 73.4 μmol ($N=3$) of the sucrose they ingested. Interestingly, there seemed to be correspondence between the amount of cane sugar each hummingbird ingested and the amount of sucrose oxidized

from these meals during the 2-h experimental period (Fig. 3, Table 3). *S. rufus* oxidized an average $31.7 \pm 2.7\%$ while *C. anna* oxidized an average of $45.5 \pm 5.9\%$ of the sucrose they ingested in the form of cane sugar. These average values were significantly different ($F_{1,5}=17.7556$, $P=0.0084$).

Discussion

Consistent with results obtained previously in rufous (Suarez et al., 1990) and broad-tailed hummingbirds (Welch et al., 2006), RQ values displayed by rufous and Anna's hummingbirds during hovering flight rapidly rose from values near 0.71–1.0 as birds transitioned from a fasted to a fed state. These results indicate that hovering hummingbirds rely largely on fatty acid oxidation to fuel hovering flight when fasted, but switch to and rely almost exclusively on carbohydrate oxidation during repeated foraging (Suarez et al., 1990; Welch et al., 2006).

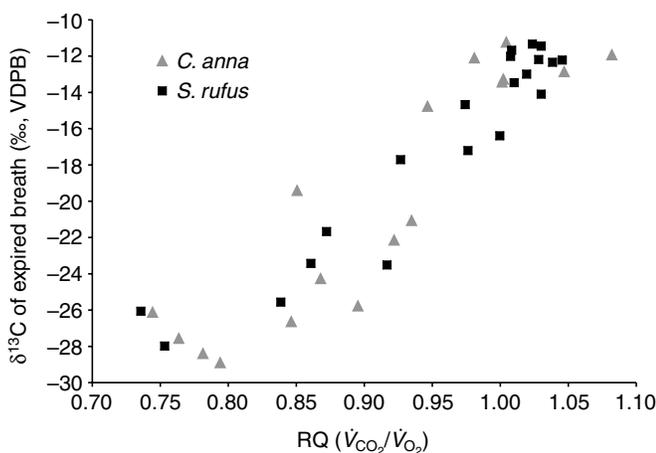


Fig. 2. Relationship between respiratory quotient ($\text{RQ}=\dot{V}_{\text{CO}_2}/\dot{V}_{\text{O}_2}$) and $\delta^{13}\text{C}$ value of expired CO_2 ($\delta^{13}\text{C}_{\text{breath}}$) during the same hover-feeding event in Anna's (*Calypte anna*) and rufous (*Selasphorus rufus*) hummingbirds. Data are pooled for each species. Pairwise comparisons reveal a significant correlation between these variables for each species (*S. rufus*: $r_{20}=0.9494$, $P<0.0001$; *C. anna*: $r_{17}=0.9065$; $P<0.0001$).

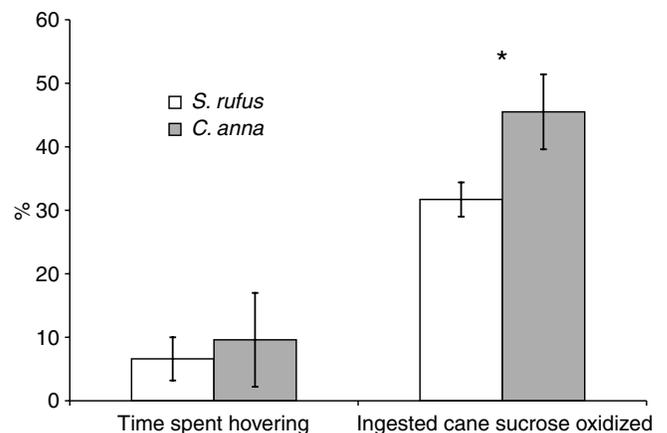


Fig. 3. Average percentage of time spent hovering and of ingested cane sugar oxidized during experiments for rufous (*S. rufus*) and Anna's (*C. anna*) hummingbirds.

Table 3. Activity records, cane sugar intake and energy expenditure for rufous (*S. rufus*) and Anna's (*C. anna*) hummingbirds

Species	ID	Sex	Mass change* (g)		Time spent hovering (%)	Total energy expenditure (J)	Cane sugar			
							Volume consumed [†] (ml)	Amount ingested (μmol)	Amount metabolized (μmol)	% Ingested oxidized
<i>S. rufus</i>										
	N43975	F	3.27	3.91	4.4	1542	0.397	232.1	72.8	31.3
	N43978	F	3.36	3.59	9.6	1637	0.909	531.4	155.2	29.2
	N44063	M	3.29	3.48	3.0	1202	0.612	357.9	109.5	30.6
	N44064	M	3.79	3.95	9.3	1820	0.483	282.3	100.4	35.6
	Mean ± s.d.				6.6±3.4	1551±259	0.601±0.224	350.9±131.0	109.5±34.3	31.7±2.7
<i>C. anna</i>										
	N44016	M	4.74	5.06	18.1	2737	0.578	501.4	244.1	48.7
	N44021	M	5.59	5.78	5.3	2363	0.372	217.4	106.8	49.1
	N44040	F	3.99	4.18	5.4	1297	0.612	338.0	130.7	38.7
	Mean ± s.d.				9.6±7.4	2132±748	0.521±0.130	352.2±142.6	160.5±73.4	45.5±5.9

*Mass change during period of cane sugar availability.

[†]Volume of cane sugar solution consumed.

Initial $\delta^{13}\text{C}_{\text{breath}}$ values indicate that hummingbirds were oxidizing endogenous energy stores derived from the maintenance diet. Respiratory quotients (RQ) associated with these initial feeding events averaged 0.74 ± 0.01 for *S. rufus* and 0.76 ± 0.02 for *C. anna*, implicating fat as the primary metabolic fuel. Average initial $\delta^{13}\text{C}_{\text{breath}}$ values were 1.18 and 1.52‰ lower than the $\delta^{13}\text{C}$ value of the maintenance diet (*S. rufus* and *C. anna*, respectively). This small discrepancy is based, in part, on the fractionation that occurs as sugars are converted into stored fat, resulting in a relative depletion of ^{13}C (DeNiro and Epstein, 1977). The magnitude of difference between the initial $\delta^{13}\text{C}_{\text{breath}}$ values and the $\delta^{13}\text{C}$ value of the maintenance diet is likely to be less than the actual degree of fractionation that occurs during fat synthesis from sugars as the initial RQ values are slightly greater than 0.71, indicating some contribution of carbohydrate (which would not be subject to the same fractionation) to the fuelling of hovering metabolism.

As hummingbirds continued to feed on the cane sugar solution, $\delta^{13}\text{C}_{\text{breath}}$ values rose towards the $\delta^{13}\text{C}$ value of the cane sugar solution and, in several individuals, actually reached this value. The increase in hovering RQ values during the period of cane sugar availability in parallel with the rise in $\delta^{13}\text{C}_{\text{breath}}$ values indicates that the source of carbohydrates being oxidized was almost exclusively dietary. That is, the rise in RQ was due almost entirely to the recruitment of newly ingested sugars into the pool of actively metabolized substrates. RQ values displayed during the subsequent period of beet sugar availability remained near 1.0, indicating a continuing reliance upon carbohydrate oxidation, despite the fact that $\delta^{13}\text{C}_{\text{breath}}$ values declined towards the $\delta^{13}\text{C}$ value of the beet sugar solution. As both cane and beet sugars consist of sucrose molecules, indistinguishable except *via* stable isotope analysis, no change in RQ would be expected as hummingbirds transitioned from reliance on one dietary sugar to the other. Thus, there was no expected significant correlation

between RQ and $\delta^{13}\text{C}_{\text{breath}}$ values during this period, and none was observed.

These results indicate that rufous and Anna's hummingbirds possess a capacity for the rapid and extensive use of recently ingested sugar in fuelling ongoing metabolism, suggesting convergence of physiological traits with other nectarivorous hovering animals such as bees and sphingid moths (Blatt and Rocas, 2001; O'Brien, 1999; Welch et al., 2006). In support of our initial hypothesis, these results are strikingly similar to those described in broad-tailed hummingbirds (Welch et al., 2006), indicating that such physiological capacities are likely widespread among small hummingbirds.

The fact that $\delta^{13}\text{C}_{\text{breath}}$ values quickly declined and approached the $\delta^{13}\text{C}$ value of beet sugar once hummingbirds were given access to this food source further supports our hypothesis that these animals make use primarily of the most recently ingested sugars when involved in steady-state foraging. As indicated by $\delta^{13}\text{C}_{\text{breath}}$ values (Fig. 1B), hummingbirds engaged in steady state foraging were no longer relying upon oxidation of cane sugar to support hovering metabolism after approximately 30 min of feeding on beet sugar. By comparison, humans exercising at approximately 45% of their maximal \dot{V}_{O_2} were observed to be still oxidizing glucose ingested more than 200 min earlier at a significant rate (Krzentowski et al., 1984). When individuals ingested glucose, rested, and then exercised at approximately 45% of their maximal \dot{V}_{O_2} , the ingested fuel remained available to the pool of actively metabolized substrates for an even greater period of time (Jandrain et al., 1984). The more rapid turnover of ingested sugars in the pool of actively metabolized substrates in hummingbirds is consistent with their small size and high mass-specific metabolic rates (Suarez, 1992).

Studies revealing net energy gain or loss in hummingbirds have traditionally relied on the monitoring of body mass over a period of several hours to several weeks (e.g. Calder et al.,

1990; Carpenter et al., 1993; Gass et al., 1999). However, studies over shorter time-scales face problems associated with smaller mass changes due to fuel storage and utilization, as well as variation in mass due to dietary water intake, and water loss. With few exceptions (e.g. Gass et al., 1999), not much can be learned when mass change is near or equal to zero.

Other methods for determining the fate of ingested carbon in birds are available. Tissues can be sampled to characterize their carbon stable isotopic signature in relation to the signature of the diet (e.g. Hobson et al., 2005; O'Brien et al., 2000; Sydeman et al., 1997; Wolf and Martínez del Rio, 2000). However, these techniques require invasive sampling that, in the case of hummingbirds, would likely be fatal and non-repeatable. On the other hand, biological ^{13}C -NMR spectroscopy for monitoring of fuel storage and metabolism requires that animals be restrained. As a result, the techniques described here are uniquely suited to the study of energy turnover in foraging hummingbirds.

Because recently ingested sugars appear and then disappear from the pool of actively oxidized substrates (as indicated by the appearance/disappearance of a characteristic $\delta^{13}\text{C}$ signature from expired CO_2), and because nearly all of ingested sugars are absorbed by the hummingbird while little is lost in excreta (Karasov et al., 1986; McWhorter et al., 2006), it is likely that sugars not immediately oxidized to support ongoing metabolic needs are stored. Although some carbohydrate is stored in the form of glycogen, it is likely that most of the excess dietary carbon is stored as fat (Carpenter et al., 1993; Odum et al., 1961; Suarez et al., 1990). By quantifying the amount of a given sugar (with a distinct isotopic signature) ingested and monitoring its rate of utilization *via* a combination of respirometry and stable isotope analysis, it is possible to determine whether sugar molecules are oxidized or stored.

Despite widely varying rates of activity, energy expenditure and rates of cane sucrose ingestion across individuals, the proportion of ingested cane sugar that was oxidized remained relatively constant within each species (Table 3). This means that within species, each individual stored the same fraction of ingested sugar despite variation in total intake. This intriguing result implies that, within species, there is relatively precise matching between each individual's rate of energy expenditure and its rate of energy intake and storage. This adds further support for the suggestion that hummingbirds possess an accurate means of matching energy intake rate to energy demand (Gass et al., 1999).

On average, Anna's hummingbirds oxidized a significantly greater proportion of ingested cane sugar than rufous hummingbirds during the 2-h experimental period ($F_{1,5}=17.7556$, $P=0.0084$). One possible explanation for the difference in the proportion of ingested energy that is oxidized as opposed to reserved for energy storage between these species lies in the differences in their life histories. The Anna's hummingbirds collected for this study were taken from a resident population at the University of California, Santa Barbara campus. Anna's hummingbirds, particularly those inhabiting coastal areas of southern and central California, tend

to stay in place in August through October (Russell, 1996) (K. Welch, personal observation), the period when our experiments were conducted. Then, they disperse after breeding in late spring and summer. On the other hand, rufous hummingbirds undergo one of the most impressive annual migrations of any animal, with some individuals migrating upwards of 6000 km from breeding grounds as far north as Alaska to wintering grounds in central Mexico (Calder, 1987; Phillips, 1975). These flights are interrupted by refuelling stops to replenish fat stores (Carpenter et al., 1983). Hiebert (Hiebert, 1993) noted that captive rufous hummingbirds maintained a higher average daily body mass during periods of the year corresponding to their southward migration compared to non-migratory times. This period of elevated body mass (mid-August through November) encompasses the period when our experiments were conducted. In contrast, Calder et al. (Calder et al., 1990) noted that resident territorial broad-tailed hummingbirds appeared to restrain food intake so as to maintain a lower body mass, presumably facilitating aerial agility. Anna's hummingbirds (adult males in particular) are territorially aggressive and may derive similar benefits from restraining mass gain during the majority of the foraging period. So, the possibility exists that rufous hummingbirds oxidized, on average, a smaller percentage of the cane sugar they ingested compared to Anna's hummingbirds, in part because of the seasonal predisposition to fat deposition. A potential application of the techniques described here is to test this hypothesis using a variety of hummingbird species with disparate life history characteristics. In contrast with other methods, the range of possibilities is considerably broadened given that the procedures can be carried out in the field.

List of abbreviations

\dot{V}_{O_2}	rate of oxygen consumption ($\text{ml O}_2 \text{ g}^{-1} \text{ h}^{-1}$)
\dot{V}_{CO_2}	rate of carbon dioxide production ($\text{ml CO}_2 \text{ g}^{-1} \text{ h}^{-1}$)
RQ	respiratory quotient ($\dot{V}_{\text{O}_2}/\dot{V}_{\text{CO}_2}$)
E	energy expenditure (J)
f_{exo}	fraction of expired CO_2 derived from oxidation of cane sugar
t	time (s)
M	amount of metabolic substrated oxidised (μmol)
Met	amount of oxygen consumed (mol)
M_b	body mass (g)
MR	metabolic rate ($\text{ml O}_2 \text{ h}^{-1}$)
$\delta^{13}\text{C}$	isotopic $^{13}\text{C}/^{12}\text{C}$ ratio referenced to international standard (Carleton et al., 2006)
VPDB	Vienna Pee Dee Belemnite C standard

Charlotte Guebels analyzed all video recordings and calculated time budgets for each hummingbird. We thank Andrea Hochevar, Andrea Wisniewski, William Talbot Bowen, Nicole Boyd, and Samantha Levinson for help in capturing and/or caring for hummingbirds. Dan Day assisted in the preparation of solid samples for submission to the

UCSB MSI Analytical Lab. We thank François Péronnet, Bradley Hartman Bakken and Carlos Martínez del Rio, who provided helpful insight and discussion regarding these data. We thank Georges Paradis and the staff at the UCSB MSI Analytical Lab for their work in analyzing all stable isotope samples. We also thank John Lighton and Sable Systems International, Inc. for equipment and technical support, and Cyril Johnson for fabrication of the IR-detector circuitry used in this study. This work was supported by NSF grant IOB 0517694 to R.K.S.

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