Carbon stable-isotope tracking in breath for comparative studies of fuel use

Kenneth C. Welch Jr., 1 François Péronnet, 2 Kent A. Hatch, 3 Christian C. Voigt, 4 and Marshall D. McCue 5

1 Department of Biology, University of Toronto Scarborough, Toronto, Ontario, Canada. 2 Département de Kinésiologie, Université de Montréal, Montréal, Québec, Canada. 3 Department of Biology, Long Island University Post, Brookville, New York. 4 Department of Evolutionary Ecology, Leibniz Institute for Zoo and Wildlife Research, Berlin, Germany. 5 Department of Biological Sciences, St. Mary’s University, San Antonio, Texas

Address for correspondence: Kenneth C. Welch Jr., Department of Biology, University of Toronto Scarborough, 1265 Military Trail, Toronto, Ontario, Canada M1C 1A4. kwelch@utsc.utoronto.ca

Almost half a century ago, researchers demonstrated that the ratio of stable carbon isotopes in exhaled breath of rats and humans could reveal the oxidation of labeled substrates in vivo, opening a new chapter in the study of fuel use, the fate of ingested substrates, and aerobic metabolism. Until recently, the combined use of respirometry and stable-isotope tracer techniques had not been broadly employed to study fuel use in other animal groups. In this review, we summarize the history of this approach in human and animal research and define best practices that maximize its utility. We also summarize several case studies that use stable-isotope measurements of breath to explore the limits of aerobic metabolism and substrate turnover among several species and various physiological states. We highlight the importance of a comparative approach in revealing the profound effects that phylogeny, ecology, and behavior can have in shaping aerobic metabolism and energetics as well as the fundamental biological principles that underlie fuel use and metabolic function across taxa. New analytical equipment and refinement of methodology make the combined use of respirometry and stable-isotope tracer techniques simpler to perform, less costly, and more field ready than ever before.

Keywords: respirometry; stable isotopes; metabolism; fuel use; CO2

Introduction and history

Tracer techniques measuring 13C in exhaled CO2 were introduced in biology between 1968 and 1973, in Liège (Belgium), following a series of observations showing that the stable carbon isotopic composition of expired breath differed among animal species. 1–3 In 1968, researchers initially speculated that this phenomenon could be due to small species-specific differences in the kinetics of the Krebs cycle and could “allow for a better understanding of the significance of systematics.” 1 Around the same time, Bender reported that the 13C content (expressed as δ13C, the difference in the 13C/12C ratio relative to a standard; see Eq. (1)) was higher in the tissues of plants that use the C4 (or Hatch–Slack cycle) than the C3 photosynthetic cycle (or Calvin cycle). 4,5 Although the groundbreaking work by Bender was not quoted in their 1971 report, Mosora et al. 6 correctly recognized that differences in breath δ13C (hereafter: δ13C CO2) among animal species were actually related to differences in the δ13C of their diets. More interestingly, both Mosora et al. 6 and Lacroix et al. 3 reported that the δ13C CO2 of rats tracked sustained changes in diet δ13C and acute ingestion of glucose.

Beginning in 1973, the correlation between the δ13C of the diet and breath in rats paved the way for the first experiments conducted in resting humans following ingestion of glucose naturally enriched in 13C (i.e., glucose derived from a C4 plant such as sugar cane or maize; δ13C VPDB = –14 to –10‰ (per mil)). 7–10 In these seminal studies, no attempt was made to calculate the actual oxidation rate...
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of exogenous glucose, but its use as an oxidative substrate was qualitatively tracked from the postabsorptive increase in $\delta^{13}$C$_{CO_2}$ over time. As expected, when compared to resting subjects with a normal glucose tolerance, exogenous glucose use was lower in patients with mild type 1 diabetes deprived of insulin for 24 h and obese subjects with mild type 2 diabetes.

In a 1976 study, researchers first quantified nutrient oxidation from measurements of $\delta^{13}$C. Mosora et al. calculated that eight normal subjects oxidized 28.6 g of a 100-g load of naturally enriched glucose over the 7 h following ingestion.\(^{11}\) The following year, Pirnay et al. reported that an entire 100-g load of naturally $^{13}$C-enriched glucose provided 24–27% of the energy expended over a 210-min period of mild exercise (~50% $V_{O_2,max}$ with $V_{O_2}$ ranging between 1.9 and 2.1 L/min).\(^{12,13}\) It should be noted that this first observation of exogenous glucose oxidation during exercise using $^{13}$C was preceded in 1973 by two studies using $^{14}$C.\(^{14,15}\) Although one of these studies failed to observe a significant oxidation of exogenous glucose (only 2.5 g over a 60-min period of exercise at 60–72% $V_{O_2,max}$), the other study reported that over a 4-h period of exercise at 47% $V_{O_2:max}$, humans oxidized 44 g of a 100-g exogenous sucrose load, accounting for 25% of their energy expenditure. In part, as a consequence of this research, a consensus regarding fuel use during moderate to intense exercise in humans and other mammals emerged: newly ingested carbohydrates can provide some of the energy needed to fuel exercise, with the majority provided by endogenous lipid and carbohydrate stores.\(^{16–18}\)

While observations of the similarities between $\delta^{13}$C$_{CO_2}$ and the diets among various animals preceded studies in humans, it took decades before the combination of respirometry and $\delta^{13}$C$_{CO_2}$ measurements were used to quantify fuel oxidation rates in other nonmodel species. Before this, stable-isotope analysis in comparative studies was generally employed to address ecological questions, correlating variation in the isotopic signature of breath or various tissues with spatial or temporal shifts in food resource use or differences among populations or trophic levels (see reviews in Refs. 19–21).

The analyses of $\delta^{13}$C$_{CO_2}$ by comparative physiologists to examine substrate oxidation gained popularity following a call for such analyses in birds by Hatch et al. in 2002.\(^{22}\) Over the next decade, a number of studies, some using a diet-switching protocol (i.e., an acute transition from a C$_1$-based to a C$_4$-based diet, or vice versa) and others relying on natural dietary variation, examined $\delta^{13}$C$_{CO_2}$ values and provided qualitative accounting of the oxidation of ingested fuels in a multitude of species (e.g., Refs. 23–31).

In a 2006 paper, Welch et al. simultaneously employed mask respirometry\(^{32}\) with $\delta^{13}$C$_{CO_2}$ analysis and an experimental design involving diet switching to quantify oxidation of exogenous sugar during hovering in broad-tailed hummingbirds.\(^{33}\) In data collected simultaneously with that reported by Welch et al. and published a year later, Voigt and Speakman employed a purely isotopic approach in a diet-switching experiment to study fuel use in nectar bats.\(^{34}\) In this and in several subsequent studies, the $\delta^{13}$C$_{CO_2}$ values of hummingbirds, sparrows, and bats were shown to rapidly approach the signature of sugar meals each had recently been fed (reviewed below).\(^{33,35–38}\) As a whole, this emerging area of study using stable-isotope tracer techniques is demonstrating that the human and mammalian paradigm of exogenous fuel use during exercise is not as broadly applicable as once thought, and that the diversity of fuel-use strategies is still a relatively unexplored area of research.

In the following review, we discuss technical issues and best practices in breath carbon stable-isotope tracking, particularly in combination with quantitative respirometry, to study fuel oxidation across taxa. We also present several case studies to underscore the power and utility of this technique and conclude by identifying several specific research areas that would benefit from using stable isotopes to track fuel use.

**Technical issues and best practices**

**Measuring and reporting $^{13}$C**

There are multiple methods to measure the ratio of $^{13}$C to $^{12}$C atoms in breath and body tissues. Solid tissue samples first need to be pyrolyzed at $>1000$ °C, generating gaseous CO$_2$ that is isolated using gas chromatography. The $^{13}$C content in breath samples can be measured directly. The gold standard and most widely employed technique for measuring the ratio of $^{13}$C to $^{12}$C is conventional isotope ratio mass spectrometry (IRMS) and involves ionizing a stream of CO$_2$ and passing the particles through an
evacuated flight tube surrounded by a strong magnetic field. Ion detectors tuned to specific particle masses located at the end of the flight tube then quantify the number of $^{12}\text{CO}_2$ (mass = 44) and $^{13}\text{CO}_2$ (mass = 45) particles in the gas stream. The ratio of the heavy to light isotope in the unknown sample is compared to an international standard (Vienna Pee Dee Belemnite; VPDB; absolute ratio of mole fraction $= 0.0112372$) and expressed in terms of $\delta^{13}\text{C}$ with units %o) according to the following equation:\textsuperscript{39}

$$\delta^{13}\text{C} = \frac{\left[(^{13}\text{C}/^{12}\text{C})_{\text{sample}} - (^{13}\text{C}/^{12}\text{C})_{\text{VPDB}}\right]}{(^{13}\text{C}/^{12}\text{C})_{\text{VPDB}}} \cdot 1000.$$ \textsuperscript{(1)}

For higher concentrations of $^{13}\text{C}$ (e.g., above 100%o), values are usually expressed in terms of atom percent (at%),\textsuperscript{40} but these two units can be interconverted using the following equations:

$$\text{at}\% = \frac{100 \cdot \text{VPDB} \left(\frac{\delta^{13}\text{C}}{1000} + 1\right)}{1 + \left[\text{VPDB} \cdot \left(\frac{\delta^{13}\text{C}}{1000} + 1\right)\right]}.$$ \textsuperscript{(2)}

$$\delta^{13}\text{C} = \frac{1000 \cdot \text{at}\%}{(100 \times \text{VPDB}) - (\text{VPDB} \times \text{at}\%)} - 1000.$$ \textsuperscript{(3)}

Modern conventional IRMS systems typically use a continuous flowing stream of helium to carry the $\text{CO}_2$ sample and thus have high costs of operation. Analyses performed by U.S. academic institutions using IRMS cost $10–20 each, depending on whether the sample is a solid or a gas. The $\delta^{13}\text{C}$ of gas samples may also be analyzed using nondispersive infrared spectroscopy (NDIS), which does not require specialized carrier gas or a strong electromagnet. This technique relies on differences in transmittance of a beam of infrared light through a gas sample. NDIS is highly sensitive to differences in gas composition, in particular the concentration of $\text{CO}_2$ in each sample, but is limited in that it requires that gas samples contain >1% $\text{CO}_2$ to approach the accuracy of conventional IRMS. Nevertheless, NDIS is well suited for clinical measurements of human breath samples that are ~4% $\text{CO}_2$\textsuperscript{41–43} and for experiments where comparably high $\text{CO}_2$ concentrations can be achieved via enrichment within a respirometry chamber.\textsuperscript{44–47}

One of the most recent advances in the measurement of $\delta^{13}\text{CO}_2$ is cavity ring-down spectroscopy (CRDS). $^{13}\text{CO}_2$, $^{12}\text{CO}_2$, and other gaseous molecules have unique near-infrared absorption spectra. CRDS works by quantifying the effect of this absorption. A laser pulse is fired into a chamber (the cavity) and reflected off a series of mirrors. As the laser bounces from mirror to mirror within the cavity, light is lost due to either absorption by the cavity walls or leakage from the chamber, as well as absorption by the gas molecules present. Because the light pulse bounces from mirror to mirror many thousands of times within the chamber, the effective path length within the cavity is several kilometers, greatly enhancing sensitivity. The absolute and relative concentration of $\text{CO}_2$ isotopic species is determined by the rate of exponential decay (the ring-down) of the light intensity. This approach does not require specialized carrier gas, and because it is capable of measuring low concentrations of $\text{CO}_2$ (e.g., 150 ppm), it can be paired with pyrolysis systems like those used for conventional IRMS. One of the greatest strengths of CRDS is that it can be paired, in line, with traditional open-flow respirometry systems. The measurement of $\text{CO}_2$ production rate is a prerequisite for quantitative accounting of fuel oxidation using the tracer techniques outlined here (see “Quantifying tracer kinetics” below). By pairing them in line, an airstream into which breath samples are introduced can be sampled sequentially by respirometry and CRDS equipment to calculate oxidation rates of an isotopically labeled tracer molecule in near real time.

**Administering $^{13}\text{C}$ tracers**

There are several ways that $^{13}\text{C}$ tracers can be administered, depending on the research question to be addressed, but they are usually orally ingested or injected into the circulation. Differences in the kinetics of orally dosed tracers with those administered directly into circulation provide insight into the extent to which exogenous nutrients are oxidized during their first pass through the splanchnic tissues.\textsuperscript{48–51} Tracers can also be administered as a single bolus or repeatedly. Single-bolus doses are useful to study the fates of dietary nutrients, while repeated exposure to tracers is required to isotopically enrich body tissues in order to examine how the body uses its endogenous macronutrients. Because the different tissues and organs each have different
turnover rates, the best way to ensure that an animal’s body is in isotopic equilibrium with its diet is to raise the animal from birth on the $^{13}$C tracers. Note that equilibrium does not mean that the isotope has become homogenously distributed throughout the body tissues. For example, the different organs and tissues of animals raised on a bulk diet with a constant $\delta^{13}$C will differ slightly in their $\delta^{13}$C.\(^{57,58}\)

When tracking the oxidation of substrates by analyzing the $\delta^{13}$C\(_{\text{CO}_2}\) of exhaled breath, it is crucial that the metabolic fate of the tracer and tracee molecules are identical. This is an acute concern when employing commercially available artificially enriched substrates. In most cases, commercially available isotopically enriched substrates are small molecules such as mono- and disaccharides, single amino acids, and fatty acids. These $^{13}$C tracer molecules can be administered in the diet with the aim of isotopically labeling a targeted macronutrient pool in the body. In order to do this effectively, the selected $^{13}$C-labeled tracer molecules should (1) not undergo extensive oxidation during the postprandial period, (2) occur in relatively high amounts in the given macronutrient pool, and (3) exhibit minimal biochemical conversion into other tissue pools. $^{13}$C-1-l-leucine, an essential amino acid and $^{13}$C-1-palmitic acid are cost-effective, widely available, and have proven to be effective tracer molecules for isotopically enriching the protein and the lipid pools, respectively, in the bodies of a variety of animals.\(^{59,60}\)

The use of $^{13}$C-glucose tracers is not recommended for long-term $^{13}$C-enrichment studies because any of the six carbon atoms could eventually become incorporated into the lipid and protein pools during \textit{de novo} synthesis of fatty acids and nonessential amino acids, respectively.\(^{60}\) Accounting for the bioconversion of $^{13}$C-glucose into other nutrient pools is possible but may not be practical for most applications.\(^{47}\)

Methods for artificially enriching more complex nutrient molecules in the laboratory setting are typically logistically impossible or prohibitively costly. When researchers want to understand the oxidative fate of more complex nutrients (polysaccharides, proteins, or triacylglycerols), the use of naturally enriched tracer molecules (starches or fats from C\(_4\) plants like corn) is preferred, as these generally exhibit uniform isotopic enrichment. It is a serious mistake to “spike” a dose of a tracee nutrient, such as starch, with an artificially enriched simpler nutrient molecule, such as glucose or succrose. As demonstrated by Saris \textit{et al.}, this approach leads to overestimation of starch oxidation because the tracer is metabolized much faster than the tracee.\(^{61}\)

In some cases, complex nutrient molecules can be enriched in $^{13}$C above levels found in nature. In the case of starch, nonnatural $^{13}$C enrichment can only be obtained by a procedure called intrinsic labeling whereby starch-producing plants are grown in an atmosphere with $\text{CO}_2$ enriched in $^{13}$C (e.g., Ref. 62). Unfortunately, the practice of intrinsic labeling has not been widely adopted.\(^{59}\)

\textbf{Intermediary metabolism and CO\(_2\) elimination}

When $^{13}$C-labeled tracers are given to animals either as part of their diets over a protracted period, infused into circulation, or fed as a single bolus, the appearance of $^{13}$C in the breath cannot be used to accurately track the intermediary metabolism of a given tracer. This is a particularly important point for studies of the fates of biochemically labile molecules like glucose, pyruvate, or acetate. If unaccounted for, natural variation in the $^{13}$C signature of endogenous nutrient pools may also confound accurate measurement of tracer kinetics. For example, feeding of naturally enriched carbohydrates (e.g., glucose derived from a C\(_4\) plant) before exercise can lead to overestimation of exogenous carbohydrate oxidation because the magnitude of the increase in $\delta^{13}$C\(_{\text{CO}_2}\) over the background value, owing to the oxidation of carbohydrate naturally enriched in $^{13}$C, is small and easily confounded with naturally occurring increases in the background $\delta^{13}$C\(_{\text{CO}_2}\) that occurs during exercise.\(^{63,64}\) This phenomenon of exercise-induced $^{13}$C enrichment in the breath is attributable to the increased contribution of endogenous glucose to energy production when transitioning from rest to exercise\(^{65}\) and to the fact that $\delta^{13}$C is slightly depleted in fat stores relative to glycogen.\(^{64,66}\)

In animals that have not been exposed to $^{13}$C tracers, the $\delta^{13}$C values of the lipid molecules in the body are isotopically lighter than those of nonlipid molecules (i.e., carbohydrates and proteins). The naturally occurring $^{13}$C depletion in lipids, which typically ranges from 5 to 15% in relation to these other nutrients and the corresponding diet, is the result of several fractionation processes, including (1) discrimination during the conversion of $^{13}$C-pyruvate to acetyl-CoA, (2) discrimination of $^{13}$C-acyl groups by carrier proteins during fatty acid
elongation, (3) discrimination of $^{13}$C-fatty acids during esterification as part of synthesis of triacylglycerols, and (4) possibly the discrimination of $^{13}$C-fatty acids during β-oxidation, although this latter process has not been as well studied as the others (reviewed in Ref. 64).

One way to minimize the confounding effects of exercise-induced breath $^{13}$C enrichment is to use substrates that are artificially enriched in $^{13}$C at much higher levels than natural abundance. With these considerations in mind, $^{13}$C-glucose tracers may provide accurate insight into oxidation rates over short time periods like tracer ingestion during exercise or during the postprandial period (Fig. 1). However, the longer the period since $^{13}$C-glucose tracer administration, the more likely it is that calculations of carbohydrate oxidation rates based on appearance of $^{13}$C in the breath will be overestimates, since some of the $^{13}$C tracer is likely to have entered other nutrient pools (e.g., fatty acids and amino acids).

Another limitation to interpreting $^{13}$C$_2$O$_2$ after administration of a $^{13}$C tracer is the mixing of $^{13}$CO$_2$ produced from its oxidation with the comparatively large circulating pool of bicarbonate ($\text{HCO}_3^-$) in the blood. A large proportion of the CO$_2$ in the body is in the form of $\text{HCO}_3^-$, and the appearance of $^{13}$C in the breath can be delayed by its residence in this pool. This delay may be negligible in active, small-bodied animals (<1 kg) that have comparatively high mass-specific metabolic rates and thus more rapid HCO$_3^-$ turnover rates, but in large animals (>5 kg), endotherms in inactive states, or ectotherms, the half-life for HCO$_3^-$ turnover can be ~1 h. Additionally, some of the $^{13}$C tracer can be lost to other carbon pools in the body via diversion of TCA cycle intermediates to nonoxidative fates, or by loss of $^{13}$CO$_2$ from the circulating bicarbonate pool to other carbon stores, such as bone carbonate.

In resting humans, approximately 20% of the $^{13}$C tracer is not recovered in exhaled CO$_2$. Thus, in such studies, a correction factor, sometimes termed a retention factor (RF), of ~0.8 is applied, so that the oxidation rate of the labeled substrate can be more accurately assessed. $^{13}$CO$_2$ retention, predominantly in the form of H$^{13}$CO$_3^-$, varies substantially as a function of organismal growth and development, nutritional state, or metabolic state. However, when oxidation rates are high, such as during exercise, or when resting metabolic rates are high relative to the size of the bicarbonate pool (such as in many small animals), $^{13}$C retention can be considered negligible over short periods. Over longer periods, the loss of $^{13}$C tracer and its secretion in nitrogenous wastes such as urea and uric acid can lead to underestimates of the actual rates of tracer oxidation. Corrections for losses of $^{13}$C tracer into nitrogenous waste can be estimated, but are best done by direct measurement of $^{13}$C in excreta.

### Quantifying tracer kinetics

In order to quantify the net rates of $^{13}$C-tracer oxidation (e.g., in terms of moles/min or mg/min), it is critical to know both the total rate of CO$_2$ production (i.e., $V_{\text{CO}_2}$) and the tracer-induced increase in δ$^{13}$C$_{\text{CO}_2}$. This increase in $^{13}$C content can be expressed as a unitless value in terms of atom fraction excess (AFE), where δ$^{13}$C$_{\text{enriched}}$ refers to the $^{13}$C in the breath or tissues of animals exposed to $^{13}$C tracers and δ$^{13}$C$_{\text{background}}$ refers to the δ$^{13}$C in the breath or tissues of the same animals before exposure to tracers or in conspecifics that have not been exposed to $^{13}$C tracers, and is calculated using the following equation:

$$\text{AFE} = \left[ \frac{\text{VPDB} \cdot \left( \left( \frac{\delta^{13}\text{C}_{\text{enriched}}}{1000} \right) + 1 \right)}{1 + \left( \text{VPDB} \cdot \left( \left( \frac{\delta^{13}\text{C}_{\text{enriched}}}{1000} \right) + 1 \right) \right)} \right] - \left[ \frac{\text{VPDB} \cdot \left( \left( \frac{\delta^{13}\text{C}_{\text{background}}}{1000} \right) + 1 \right)}{1 + \left( \text{VPDB} \cdot \left( \left( \frac{\delta^{13}\text{C}_{\text{background}}}{1000} \right) + 1 \right) \right)} \right].$$

(4)

The molar rate of tracer oxidation ($T$) at any time point can then be calculated according to the following equation:

$$T = \left[ \frac{V_{\text{CO}_2} \cdot \text{AFE}}{m \cdot \theta \cdot \text{RF} \cdot K} \right].$$

(5)

where $m$ is the molar mass of the tracer, $\theta$ is the number of $^{13}$C atoms in each tracer, RF is the term that corrects for the loss of label to other carbon pools (see above), and $K$ is the volume of CO$_2$ produced per unit mass of tracer oxidized (e.g., in milliliter CO$_2$/mg). Values for $K$ can be calculated on the basis of the known stoichiometry of substrate oxidation (e.g., 6 moles of CO$_2$ produced per mole glucose oxidized) and the volume occupied per mole of CO$_2$ gas (~22.4 L).

$T$ can be plotted over time and the integral of the function can be expressed as cumulative
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Figure 1. The \(\delta^{13}\text{C}\) of the breath of Japanese quail after being administered an oral gavage of a mixed meal (i.e., carbohydrate, lipid, and protein) containing equal amounts of \(^{13}\text{C}\) atoms that were either intrinsically labeled (i.e., in the forms of \(^{13}\text{C}\)-ovalbumin or \(^{13}\text{C}\)-tripalmitin) or extrinsically labeled (i.e., in the forms of \(^{13}\text{C}\)-leucine or \(^{13}\text{C}\)-palmitic acid). In both cases, the \(\delta^{13}\text{CO}_2\) was higher after ingesting the extrinsically labeled tracer, underscoring the fact that extrinsic-labeled tracers may not be an accurate proxy for the oxidative fates of larger macronutrients. Data presented as mean ± SD and are modified from Ref. 144.

oxidation (in moles) or further converted to percent dose recovered (PDR). It is noteworthy that PDR is likely to be dose dependent, and therefore caution should be used when comparing PDR values between studies. Nevertheless, studies of post-prandial birds, bats, and rodents have shown that exogenous amino acids and simple sugars are oxidized at rates an order of magnitude greater than fatty acids.\(^{76,77}\)

Issues of atmospheric dilution of the breath
The atmosphere contains approximately 0.04% \(\text{CO}_2\), and has a \(\delta^{13}\text{C}\) of \(-8\%_o\) to \(-10\%_o\). Because of the biochemical discrimination of atmospheric \(^{13}\text{CO}_2\) in plants by the enzyme ribulose-1,5-bisphosphate carboxylase/oxygenase (RuBisCO),\(^{78,79}\) ambient \(\text{CO}_2\) is significantly more enriched in \(^{13}\text{C}\) relative to the exhaled breath of most animals, even those animals raised on a diet based on \(\text{C}_4\) plants. If the \(\text{CO}_2\) from the breath is allowed to mix with the \(\text{CO}_2\) in the air, it could preclude accurate measure of exhaled \(\delta^{13}\text{CO}_2\). The concentration of \(\text{CO}_2\) in a pure breath sample of a human is \(~4\%\) (100 times atmospheric concentration), and thus the effect of atmospheric \(\text{CO}_2\) dilution on \(\delta^{13}\text{CO}_2\) is negligible. However, when exhaled breath is highly diluted in
air, for example when air is pumped into a metabolic chamber during open-flow respirometry, the effect of atmospheric dilution can become significant.

Mathematical mixing models can be used to correct for atmospheric dilution, since the CO$_2$ concentration and the $\delta^{13}$C$_{\text{CO}_2}$ in the air are relatively constant. Unfortunately, because the concentration of exhaled CO$_2$ is subject to change with tidal volume, breathing frequency, and metabolic rate, quantification of total CO$_2$ in analyzed samples is necessary to ensure accurate correction. This is possible using closed-system respirometry approaches and subsampling of (atmospherically diluted) breath samples. When background atmospheric air samples are included with collected (atmospherically diluted) breath samples, mixing models have been used with success (e.g., Refs. 24, 33, 35, and 38). Dynamic mixing models that incorporate changes in the $V_{\text{CO}_2}$ of animals may be possible using CRDS systems, but we are not aware of any studies that have done this yet. Other approaches can eliminate the need for correction factors altogether. For example, small volumes (e.g., <2 L/min) of CO$_2$-free gas can be generated by passing air through chemical scrubbers (e.g., soda lime) to remove the CO$_2$ whereas larger volumes (e.g., >50 L/min) of CO$_2$-free air can be generated using purge-gas generators, thereby eliminating the issue of atmospheric CO$_2$ contamination. However, if CO$_2$ levels in diluted breath samples are sufficiently high (e.g., >1%) and/or when using large $\delta^{13}$C enrichments (e.g., >100%) the effect of atmospheric dilution may be negligible (e.g., Ref. 80).

**Case studies**

**Exogenous sugar oxidation in humans**

Since the pioneering works by Benadé et al. and Pirnay et al., more than 125 studies have described the oxidation of various exogenous substrates ingested during exercise in humans, mainly using $^{13}$C. Among all energy substrates that have been compared when ingested alone, the highest oxidation rate during exercise is observed for glucose (up to 1 g/min for ingestion rates $>\sim 2$ g/min). When compared with glucose, under similar conditions, the oxidation rate of maltose (a glucose dimer) or of glucose polymers of different molar masses are similar, provided that these are not resistant to digestion. Exogenous glucose is consistently oxidized at a higher rate than other carbohydrates, including fructose, galactose, isomaltulose, trehalose, and lactate. However, when mixtures of various carbohydrates are ingested, under similar conditions and in similar amounts, the highest oxidation rates are observed with a mixture of glucose and fructose. This outcome is probably due to the fact that glucose and fructose do not compete for absorption across the apical membrane of the enterocyte but can be absorbed, at least in part, through distinct mechanisms: transport by SGLT1 for glucose (also responsible for galactose absorption) and GLUT5 for fructose. Since sucrose is the equivalent of a mixture of glucose and fructose, for similar amounts ingested, the oxidation rate of sucrose has not surprisingly been shown to be higher than that of glucose alone (Fig. 2).

From a practical point of view, an extensive comparison of the oxidation rate of mixtures of “multiple transportable carbohydrates” performed by Jeukendrup and colleagues shows that the highest oxidation rates (close to 1.8 g/min) were observed for mixtures of glucose and fructose or of glucose, fructose, and sucrose, with a 2.4 g/min ingestion rate. This maximal value for exogenous glucose compares well with the maximal rate of plasma glucose oxidation reported by Hawley et al. in subjects exercising during a hyperglycemic clamp with plasma glucose value $\sim 10$ mmol/L ($\sim 1.9$ g/min) and indicates that the flux of glucose from the mouth to the exercising muscle is an example of economic design or symmorphosis.

It is worth noting that some of the higher carbohydrate doses (and the corresponding oxidation rates) described previously are well above those at which the ergogenic effects of carbohydrates on endurance performance are observed, which range between only 0.4 and 1.1 g/min (corresponding to an oxidation rate of only $\sim 0.3$–0.7 g/min.) However, the ingestion of large amounts of carbohydrates can be useful for athletes engaged in prolonged exercise repeated for several days in a row such as multi-day bicycle races. For example, Saris et al. collected data on cyclists during the Tour de France—approximately 4000 km in 22 days with an average speed close to 40 km/h, corresponding to an average daily exercise time of $\sim 270$ min mainly at moderate workload ($\sim 45$–55% $V_{O_2}^{\text{max}}$ except during climbing and time trials) for athletes with a very high $V_{O_2}^{\text{max}}$ (see Ref. 98 for review). The
average amount of carbohydrates ingested during a stage corresponds to \( \sim 520 \) g of hexoses or almost \( 2 \) g/min, mainly in the form of glucose. Assuming that \( \sim 50\% \) of this dose is actually oxidized or \( 1 \) g/min, exogenous carbohydrates oxidation can provide \( \sim 20\% - 30\% \) of the required energy. This strategy not only spares endogenous substrates, but also ensures the amount of nonoxidized exogenous carbohydrates ingested is available to restore glycogen stores as they are utilized, a phenomenon described by Kuipers et al.\(^{99} \). Interestingly, hummingbirds and nectarivorous bats may naturally use a similar strategy (see below).

**Glucose and fructose oxidation in hovering vertebrate nectarivores**

Unlike humans, vertebrate nectarivores like hummingbirds and New World nectar bats subsist almost exclusively on a nectar diet that is 50\% glucose and 50\% fructose (with varying amounts of these packaged as the disaccharide sucrose).\(^{100,101} \)

Both hummingbirds and nectar bats also employ energetically intensive hovering flight, achieving mass-specific oxygen consumption rates many times greater than humans and other nonvolant vertebrates.\(^{36,102-104} \) Such high metabolic rates demand abundant, readily available oxidative fuels. Yet, hummingbirds and nectar bats face potential challenges in building the large intramuscular glycogen stores that sustain humans during exercise. Glycogen is a hydrophilic molecule, and provides approximately 10\% the energy per unit wet mass of lipids.\(^{105,106} \) Thus, energy dense, hydrophobic lipid stores represent a weight-saving fuel of choice for nonforaging or migrating hummingbirds and nectar bats.\(^{107-111} \)

Foraging hummingbirds and nectar bats could convert ingested sugars into lipids to replenish lipid stores in an ongoing fashion. However, this strategy would involve a roughly 15\% loss of the caloric energy in ingested nectars compared to their immediate oxidation.\(^{112} \) The alternative, fuel energetically intense foraging activity (such as hovering flight) completely with ingested sugar, does not occur in nonvolant vertebrates and would mean that these nectarivores possess dramatically different physiological capacities for the absorption and transport of sugar and its uptake and oxidation by flight muscle fibers.

Beginning in 2006, Welch et al. used mask respirometry in combination with \( \delta ^{13} \text{C}_{\text{CO}_2} \) analysis and a diet-shifting protocol to demonstrate that hummingbirds could fuel most or all of hovering flight with nectar ingested within the previous 30 min.\(^{33,35,38} \) After 30–50 min, rufous (Selasphorus rufus), Anna’s (Calypte anna), and ruby-throated

**Figure 2.** The percentage of exercise (humans: stationary cycling; birds/bats: hovering flight) metabolism fueled by the oxidation of exogenous sugar (> 30–60 min after initial feeding). Humans were fed either glucose, fructose, or a glucose + fructose solution (2:1 ratio). Hummingbirds and bats were fed glucose, fructose, or sucrose solutions. Data are presented as mean ± SEM and are from Refs. 84 (human: glucose and fructose), 145 (human: glucose + fructose mixture), 36 (bats, Anna’s, and rufous hummingbirds), and 38 (ruby-throated hummingbirds).
hummingbirds (Archilochus colubris) were supporting 95 ± 2%, 96 ± 2%, and 92 ± 6% (mean ± S.E.M.), respectively, of hovering metabolism with exogenous sucrose (Fig. 2).36,38 Remarkably, when the newly offered nectar was replaced with nectar exhibiting the same 613C value as the maintenance diet, the 813C CO2 returned just as rapidly to that of the most recently ingested meal. Calculations revealed that the half-life of availability of ingested carbon within the pool of actively metabolizable substrates was approximately 20 min.35,38 Averaged across 20 min of hovering and intervening perching behavior, hummingbirds oxidized exogenous sucrose at a rate of 2–6 μmol hexose/g muscle/min, a mass-adjusted rate that is five to seven times greater than that seen in nonvolant vertebrates during intense exercise.113 Assuming the hummingbirds’ flight muscles were taking up and oxidizing exogenous sugar at rates sufficient to sustain ongoing oxidative demand by fibers, the calculated uptake and oxidation rates during exercise were an astounding 12–17 μmol hexose/g muscle/min.113

Two research groups using breath-testing approaches demonstrated that nectar bats possessed a similar capacity to rapidly transition to supporting activity with newly ingested nectar.34,36 After 30 min, nectar bats were supporting 78 ± 5% of hovering metabolism with newly ingested sucrose (Fig. 2).36 This translated to a sugar oxidation rate during hovering of roughly 8 μmol hexose g/muscle/min, a value somewhat lower than that observed in small hummingbirds, but still roughly three times greater than that seen in nonvolant mammals, including human athletes (Fig. 2).113

While ingestion of glucose/fructose mixtures can push reliance on exogenous sugars during exercise above levels seen when only glucose is ingested in humans,17 almost all of the ingested fructose must first be converted into glucose in the liver and then re-released into circulation before uptake (as glucose) and oxidation by muscles.114,115 Given the preference of nonvolant vertebrate skeletal muscle for glucose as an oxidative fuel over fructose, it seemed possible that this component of the diet of hummingbirds and nectar bats was preferentially oxidized. As with the Tour de France cyclists mentioned previously, animals can use ingested carbohydrates to fuel ongoing foraging (or exercise) while simultaneously reserving some for conversion to energy stores for later use during nonforaging periods. Thus, the preferential use of dietary glucose to fuel ongoing metabolism, with fructose preferentially serving as a substrate for lipogenesis, could represent an optimal fuel use strategy for foraging vertebrate nectarivores. Yet, when administered either isotopically labeled glucose or fructose solutions alone, both hummingbirds and nectar bats displayed exogenous sugar oxidation kinetics nearly identical to those observed when they were offered sucrose (Fig. 2).34,38 This evidence suggests that hummingbirds and nectar bats can make use of circulating fructose as an oxidative fuel in locomotor muscles at rates equal to the use of circulating glucose. If confirmed, this would represent a truly novel ability among vertebrates. Further, it would suggest that specialization on a diet equally rich in fructose and glucose, unlike many vertebrate diets, has dramatically shaped fuel-use strategies. Hummingbirds and nectar bats behave as carbohydrate maximizers regardless of the nectar sugar involved.

Many questions remain about how each group maximized capacities for both glucose and fructose uptake from circulation and oxidation in muscle. A key transporter in the uptake of glucose into vertebrate skeletal muscle, insulin and exercise-responsive glucose transporter isoform 4 (GLUT4), is not present in hummingbirds or any avian lineage.116,117 Thus, at least some aspects of the pathway for and regulation of sugar flux from the diet to exercising flight muscle, though convergent in overall function, is distinctly different in hummingbirds and nectar bats.

**Fuel use during long duration and migratory flight in birds**

Flight can be one of the most energetically demanding forms of locomotion, yet flight can also permit birds to cover large distances over a short period of time at lower weight-specific costs of transport (in terms of J/m/kg) than walking or running (reviewed in Ref. 118). During fall migration, great snipes fly nonstop up to 6800 km from Scandinavia to sub-Saharan Africa, and a bar-tailed godwit was documented flying nonstop from Alaska to New Zealand, a distance of 11,690 km.119 During such long migratory flights, when birds are essentially fasting, flight must be fueled entirely from endogenous resources. As mentioned above, lipid stores are an important endogenous fuel source for such journeys because of their high energy density.
Unlike hummingbirds, the vast majority of migratory birds subsist on diets considerably more complex than sugar-rich floral nectar. While many birds consume carbohydrate-rich foods, these carbohydrates are instead often found in the form of starch. It is unknown to what extent these birds rely on endogenous versus exogenous sources to fuel flight.

Taking advantage of the fact that lipid molecules are naturally depleted in $^{13}$C relative to proteins and carbohydrates, Hatch et al. tracked both blood metabolites and the $\delta^{13}$C$_{CO_2}$ of exhaled breath to compare endogenous substrate metabolism of resting control tippler pigeons and fasted tippler pigeons immediately after 4-h flights. The $\delta^{13}$C$_{CO_2}$ of birds fasted for only 2 h before flight revealed that endogenous carbohydrates primarily fuel the first few hours of flight, whereas birds that were fasted 24 h or longer fueled flight predominantly with lipids. Interestingly, whereas the blood uric acid levels were highly variable, the $\delta^{13}$C$_{CO_2}$ indicated that the ratio of lipid to protein metabolism remained constant for both resting and flying birds fasting 24–72 h, supporting the hypothesis of Jenni and Jenni-Eiermann that protein and lipid metabolism in birds are closely tied through the citric acid cycle. For example, when glycogen stores are low and fatty acid is being catabolized for flight, intermediates within the citric acid cycle are continually lost and need to be replaced. In the absence of glycogen, amino acids from endogenous proteins must supply these intermediates (anaplerosis). Thus, the oxidation of lipids appears to require the concomitant metabolism of a proportional amount of protein to maintain the citric acid cycle (See Ref. 105 and sources therein).

**Fuel use during fasting and starvation**

Food limitation presents an omnipresent physiological challenge for animals. The long-standing paradigm is that fasting animals pass through three sequential physiological phases whereby they predominantly oxidize endogenous fuels in the order of carbohydrates, then lipids, and then proteins. Historically, the timing of these metabolic transitions have been based on measurements of circulating metabolites, respiratory exchange ratio ($RER = V_{CO_2}/V_{O_2}$), or differential changes in body mass; but each of these measures carries inherent drawbacks that may preclude an accurate characterization of how animals ration their limited resources during fasting. In particular, altered levels of blood metabolites preclude inferences as to whether a given metabolite is either being synthesized (or liberated) or degraded (or excreted) at changing rates. Changes in $RER$ are often too variable to interpret quantitatively and may also be confounded with shifts in acid–base balance. Changes in mass loss can be confounded with overall hydration status as well as the general increase in the moisture content of tissues and organs that usually takes place during prolonged fasting.

Recent computer models have shown that the classic three phases of nutrient use during food limitation may not be bioenergetically optimal and do not necessarily maximize individual total starvation tolerance. Furthermore, the strategy of oxidizing carbohydrates, followed by lipids, and finally proteins may actually decrease Darwinian fitness, either by increasing the risk of predation or by limiting future reproductive success. As such, it might be reasonable to expect that the way that fasting animals alter their oxidative fuel mixtures is shaped by their unique life histories and ecology and must thus be highly variable among species. In fact, recent studies using $^{13}$C tracers have shown that some species do not follow predictions of the classic three-phase model.

If the different macronutrient pools of the body (i.e., carbohydrates, lipids, and proteins) have unique $^{13}$C-values, then measurements of the $\delta^{13}$C$_{CO_2}$ can be used to infer the extent to which a fasting animal is altering its fuel mixture. These changes can be observed in wild animals containing natural abundance levels of $^{13}$C or in laboratory animals whose tissues have been artificially enriched in $^{13}$C by feeding them special diets. Below, we review examples of how $\delta^{13}$C$_{CO_2}$ measurements can be used to characterize fasting-induced changes in fuel oxidation.

Given that lipid stores are naturally 5–15% depleted in $^{13}$C relative to other potential fuels like carbohydrate and protein, decreases in $\delta^{13}$C$_{CO_2}$ values should reflect increases in reliance on lipid versus nonlipid substrates during fasting. In a recent experiment, rats were raised on a standard diet and were fasted until they lost 30% of their initial body mass. Their $\delta^{13}$C$_{CO_2}$ values became depleted by ~3% over the first 48 h of fasting, indicative of a transition away from carbohydrate and toward lipid...
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Figure 3. The $\delta^{13}\text{CO}_2$ of ($n = 12$) adult laboratory rats fasting until they lost 30% of their initial body mass. The decrease in $\delta^{13}\text{CO}_2$ during the first 2 days corresponds with a reduction in carbohydrate oxidation and increased lipid oxidation. The gradual increase in $\delta^{13}\text{CO}_2$ corresponds with increased protein oxidation. Data presented as mean $\pm$ SD and are modified from Ref. 136.

Animals whose tissues have been artificially enriched in $^{13}\text{C}$ can also provide new information about fuel use during prolonged fasting. Two otherwise identical populations of juvenile animals can be raised in parallel to adulthood on diets enriched with $^{13}\text{C}$-leucine or $^{13}\text{C}$-palmitic acid to ensure that their proteins or lipids, respectively, are enriched with $^{13}\text{C}$. The $\delta^{13}\text{C}_2$ can then be measured before and during fasting, and the changes in the $\delta^{13}\text{C}_2$ can be used as a proxy for protein and lipid oxidation. This approach has revealed previously undocumented circadian changes in fuel oxidation during fasting in mice and quail (Fig. 4A and B) and the dramatic differences in starvation strategies between insects belonging to the same family (Fig. 4C and D).46,47

The oxidative fuel of high and low metabolism in bats

While not all bats subsist on a nectar-rich diet, all bats employ energetically intense powered flight. Since flight metabolism is about 15 times higher than resting metabolism, selection has likely acted strongly on efficient pathways for the provisioning of muscle fibers with oxidative fuels in this entire

Figure 4. The fasting-induced changes in $\delta^{13}\text{CO}_2$ of populations of (A) laboratory mice, (B) Japanese quail, (C) house crickets, and (D) lubber grasshoppers raised on either $^{13}\text{C}$-enriched amino acids or fatty acids to $^{13}\text{C}$-enrich either the protein or the lipid pools of the body, respectively. The dotted line represents the initial, prefasting $\delta^{13}\text{C}$ values in the breath of each population. Note that although the animals generally increased lipid oxidation at the start of fasting, they show varied strategies for oxidizing their endogenous macronutrients as fasting progresses. Data are modified from Refs. 46, 47, and 146.
Perhaps not surprisingly, fruit-eating bats, such as Seba’s short-tailed fruit bat, *Carollia perspicillata*, like their nectar-eating counterparts described above, can make rapid use of the sugar in their diet as an oxidative fuel (Fig. 5). Like in hummingbirds, the turnover of newly ingested sugar is also rapid, as can be inferred from the rapid decline of $^{13}$C tracer in the exhaled breath. Interestingly, when *C. perspicillata* are allowed to fly for a short period (1 min) about 30 min after having been fed $^{13}$C-enriched glucose, the $^{13}$C enrichment of exhaled breath increases from about 0.043 AFE to 0.050 AFE ($n = 5$). This finding suggests that, during the onset of flight, bats even increase reliance on exogenous carbohydrate relative to endogenous sugars such as glycogen, a finding that seems to be counterintuitive given that endogenous glycogen is presumed readily available in the muscle fiber. The rapid transition to reliance on exogenous sugars involves upregulation at multiple points along the sugar oxidation cascade, the pathway for sugar transport from the gut to the muscle mitochondria.

Migratory bats, such as the insectivorous *Pipistrellus nathusii*, face different physiological challenges than nonmigratory nectar or fruit specialists. Since migratory bats fly continuously for long periods, they cannot depend largely on...
endogenous glycogen as an oxidative fuel. Instead, they may use exogenous nutrients from insects that they catch en route, or from endogenous lipids. In a series of experiments, Voigt et al. fed *P. nathusii* 13C-enriched amino acids or 13C-enriched fatty acids and measured the δ13C of postabsorptive animals. Since the 13C tracer was only found in the exhaled breath of bats fed 13C-enriched glycine but not in those fed 13C-enriched palmitic acids, the authors inferred that migratory bats oxidize proteins of hunted insects directly, but route the fat portion of insects to fat stores.141 The direct oxidation of proteins has also been confirmed for nonmigratory insectivorous bats.31 Similar patterns are seen in other insectivorous or sanguinivorous bats that do not depend on a carbohydrate-rich diet, with δ13C values matching the isotopic composition of their recently ingested prey.30,31 In fasting bats, like in the fasting rats described above, the δ13C of CO2 is usually depleted in 13C with respect to their normal diet, suggesting that lipids are the preferred endogenous fuel during these periods.23,29,30,34,44 Overall, the direct oxidation of exogenous nutrients while flying seems to be a general feature of bats and can best be described as aerial refueling.31,139,141

Conclusions and future directions

Compared to the more than 125 studies that have used stable (and in some cases, radio-) isotope analysis to track and quantify exogenous fuel use in humans, comparative research among other animal groups is still in its infancy. As studies on birds, bats, and even invertebrates have shown, there is broad diversity in the fuel-use strategies employed throughout the animal kingdom waiting to be described using these approaches. Already we will have to reconsider long-held assumptions that mammals and other vertebrates are constrained to fueling intense muscle activity primarily with intra-muscular glycogen. We are beginning to learn that capacities for the utilization of recently acquired proteins, lipids, and even fructose vary more profoundly than previously appreciated. Recent work is also shedding new light on the complex balance of metabolic fuels utilized even under conditions of food limitation. The case studies reviewed above highlight the wonderful diversity of fuel-use strategies that exist among animals. What shapes the evolution of these distinct fuel-use strategies can tell us a great deal about how malleable, adaptive, and resilient metabolic physiologies can be. Apart from a few select species, the influences of size, dietary ecology, behavior, and evolutionary background on patterns of fuel use remain poorly understood.

Stable-isotope analysis offers numerous advantages over other approaches, particularly in field settings and with species of special concern. Unlike radio-labeled tracers, the administration of stable isotopes involves no inherent health concerns and requires no additional permission beyond that already required to work on vertebrates or wild-caught animals. Additionally, with the introduction of new technologies such as CRDS, analysis of samples is simpler and (after the initial capital investment) less costly than ever.

The tracking of δ13CO2, following feeding with a 13C-labeled tracer is likely the simplest approach to implement on a wider scale. Coupled with chamber respirometry, δ13CO2 analysis can be used on virtually any species to quantify exogenous fuel oxidation rates at rest. With the addition of mask respirometry, exogenous fuel oxidation rates during exercise can be examined in animals running on treadmills, or flying in wind-tunnels.

δ13CO2 analysis as a tool to quantify the kinetics of fuel oxidation in animals in other physiological states beyond those summarized here has not yet been broadly attempted. Much could be gained in the study of fuel use during hypometabolic states, such as hibernation or torpor, or when animals adopt heterothermic behaviors.77 Similarly, stable isotope analysis can complement existing lines of research examining metabolism and energetics in animals facing thermal stress, dehydration, or immune challenges. Coupled with tissue analysis, δ13CO2 analysis could be used to study catabolic versus anabolic routing of carbon and other isotopes in growing or pregnant/ gravid animals.

Measures of δ13CO2 can successfully be combined with other isotopic techniques, such as the doubly labeled water (DLW) method, to reveal new insights into fuel use and foraging and locomotor behavior. The DLW method involves injection of water labeled with 18O and deuterium and subsequent sampling in order to evaluate metabolic rate over periods of several hours to several days in freely behaving animals (reviewed in Ref. 142). Its minimally invasive nature means that, like breath stable-isotope analysis, it is amenable to field studies on various animals. For example, many
migratory birds, including the tiny ruby-throated hummingbird (≈3 g) engage in specific premigratory fattening periods to build endogenous energy stores before long-distance flights. DLW and breath stable-isotope analyses could be combined to study how birds behaviorally or physiologically modulate the use of exogenous versus endogenous energy stores during premigratory periods to optimize weight gain. Alternatively, these complementary approaches could be used to understand when and why migrating birds or other animals prioritize flight speed over the efficiency of endogenous fuel use.

The use of stable-isotope analysis to study fuel use in diverse animal groups will also benefit by adopting additional, combined approaches pioneered in human studies. For example, by coupling blood sampling with δ13C CO2 analysis following feeding with a 13C-labeled carbohydrate, researchers can track not only the oxidation of exogenous fuels, but also the relative contributions of circulating glucose (e.g., from liver glycogen stores and gluconeogenesis) and intramuscular glycogen to fueling metabolism. The analysis of δ13C CO2 and 15N/14N in excreted ammonia, uric acid, or urea can be used to examine the oxidation of amino acids. Finally, the recent broader availability of automated compound-specific stable-isotope analysis makes it increasingly feasible to examine the bioconversion of metabolites and the fates of individual carbon atoms within labeled substrates.

We sit on the precipice of a golden age in the comparative study of fuel use facilitated by the use of naturally and artificially stable isotopically labeled tracers. Careful, calculated execution of stable-isotope analysis in comparative studies promises to reveal fundamental insights into metabolic physiology, the fueling of behavior, and how these have been shaped by ecological and evolutionary forces.

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Conflicts of interest
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References
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