J Comp Physiol B (2016) 186:265–285
DOI 10.1007/s00360-015-0950-4

REVIEW

^{13}C-Breath testing in animals: theory, applications, and future directions

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Received: 3 September 2015 / Revised: 11 November 2015 / Accepted: 25 November 2015 / Published online: 11 December 2015

Abstract The carbon isotope values in the exhaled breath of an animal mirror the carbon isotope values of the metabolic fuels being oxidized. The measurement of stable carbon isotopes in carbon dioxide is called ^{13}C-breath testing and offers a minimally invasive method to study substrate oxidation in vivo. ^{13}C-breath testing has been broadly used to study human exercise, nutrition, and pathologies since the 1970s. Owing to reduced use of radioactive isotopes and the increased convenience and affordability of ^{13}C-analyzers, the past decade has witnessed a sharp increase in the use of breath testing throughout comparative physiology—especially to answer questions about *how* and *when* animals oxidize particular nutrients. Here, we review the practical aspects of ^{13}C-breath testing and identify the strengths and weaknesses of different methodological approaches including the use of natural abundance versus artificially-enriched ^{13}C tracers. We critically compare the information that can be obtained using different experimental protocols such as diet-switching versus fuel-switching. We also discuss several factors that should be considered when designing breath testing experiments including extrinsic versus intrinsic ^{13}C-labelling and different approaches to model nutrient oxidation. We use case studies to highlight the myriad applications of ^{13}C-breath testing in basic and clinical human studies as well as comparative studies of fuel use, energetics, and carbon turnover in multiple vertebrate and invertebrate groups. Lastly, we call for increased and rigorous use of ^{13}C-breath testing to explore a variety of new research areas and potentially answer long standing questions related to thermobiology, locomotion, and nutrition.

Keywords Bioenergetics · Metabolism · Metabolic tracers · Metabolic fuel · Exercise · Flight · Disease · Fatty acids · Lipid metabolism · Amino acids · Protein metabolism · Carbohydrate metabolism · Digestion · Nutrition · Starvation · Disease

Introduction

The use of ^{13}C-breath testing in the field of comparative physiology has greatly expanded over the past decade and promises to continue rapidly growing. The aim of this review is to summarize the basic physical and biochemical principles that underlie the general use of breath testing and to synthesize recent studies employing breath testing in human and animal models. We use specific examples to underscore the diverse range of research questions that breath testing can be used to answer and identify the strengths and weaknesses of different approaches. We dedicate the final section to identifying unanswered questions in comparative physiology that may finally be addressed using this powerful approach.

Reporting isotope concentration

Elemental carbon naturally occurs in three isotopic forms. The two most common isotopes (^{12}C and ^{13}C) are stable and not radioactive like ^{14}C. Almost 99 % of the carbon in the earth's crust is ^{12}C, and its heavier isotopic sibling (^{13}C) accounts for most of the remaining 1 % (De Laeter et al.

Communicated by I. D. Hume.

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2003). ^{13}C is not homogenously distributed in the earth's crust and its relative abundance differs among major carbon pools such as the atmosphere, fossil fuels, ocean bicarbonate, and living organisms (De Laeter et al. 2003; Fry 2006). Fortunately researchers are able to accurately measure the minuscule differences in the relative and absolute ^{13}C-content across this isotopic landscape.

The amount of ^{13}C in a given sample (solid, liquid, or gas) is traditionally expressed in terms of a *delta value* that is calculated as a molar ratio of the ^{13}C to ^{12}C in the sample compared to an international standard material (Vienna Pee Dee Belemnite; V-PDB; absolute ratio of mole fraction = 0.0112372) and expressed in terms of $\delta^{13}C$ with units ‰ (per mil) according to the Craig equation (Craig 1957).

$$\delta^{13}\mathrm{C} = \left[\frac{\left(^{13}\mathrm{C}/^{12}\mathrm{C}\right)_{\mathrm{sample}} - \left(^{13}\mathrm{C}/^{12}\mathrm{C}\right)_{\mathrm{VPDB}}}{\left(^{13}\mathrm{C}/^{12}\mathrm{C}\right)_{\mathrm{VPDB}}}\right] \times 1000 \quad (1)$$

Other variations of nomenclature (e.g., Urey; Ur) that more closely parallel the requirements for SI units have also been proposed (Brand and Coplen 2012). For higher concentrations of ^{13}C (e.g., above 100 ‰) these concentrations are more properly expressed in terms of atom percent (At %) (Slater et al. 2001), but these two units can be interconverted using the following equations (Fry 2006; Welch et al. 2015).

$$\mathrm{At}\ \% = \left[\frac{100 \times \mathrm{VPDB}\left(\frac{\delta^{13}\mathrm{C}}{1000} + 1\right)}{1 + \left[\mathrm{VPDB} \times \left(\frac{\delta^{13}\mathrm{C}}{1000} + 1\right)\right]}\right] \quad (2)$$

$$\delta^{13}C = \left[\frac{1000 \times \mathrm{At}\ \%}{(100 \times \mathrm{VPDB}) - (\mathrm{VPDB} \times \mathrm{At}\ \%)}\right] - 1000 \quad (3)$$

For consistency, we will hereafter refer to the ^{13}C-content in a material in terms of $\delta^{13}C$.

History

^{13}C-breath testing (hereafter: breath testing) is an approach where researchers measure the $\delta^{13}C$ in the exhaled carbon dioxide of air-breathing animals (Fig. 1). Because CO_2 is a waste product of the oxidation of organic compounds in the body breath testing can be used to study a variety of metabolic and physiological processes. Before the 1980s the use of radioactive, ^{14}C-tracers predominated studies of human and animal metabolism in vivo (Matthews and Bier 1983; Patterson and Veillon 2001). Radioisotopes could be measured quite cheaply using scintillation counting, but the costs of disposing of the radioactive waste products (e.g., old samples and animal tissues) have substantially increased over the years making their use less practical (Rennie 1999). Other logistical factors have also contributed to the displacement of radioactive carbon tracers. For example, small-scale use of ^{13}C-tracers does not require specialized permitting and oversight by regulatory bodies like the Nuclear Regulatory Commission in the US. From a health perspective, stable isotopes are preferred when working on pediatric subjects (Bier 1997; Bodamer and Halliday 2001; Mahon and Timmons 2014) or animals that must be returned to the wild (Pauli et al. 2009; Voigt 2009). Stable isotope samples are also more convenient because they can be stored without concern of tracer decay. In fact ^{13}C-breath samples can be stored at room temperature for several weeks (McCue et al. 2010b; Passey et al. 2005) without measurable changes in the $\delta^{13}C$ values.

Natural abundance or artificially enriched

Breath testing studies may be divided into one of two categories—natural abundance or artificially enriched—and their use in comparative physiology is rooted in two larger research areas. In particular, breath testing of natural abundance levels (i.e., naturally occurring variation in relative concentrations) branched from the field of isotope ecology (sensu Martinez del Rio et al. 2009; Newsome et al. 2007; Thompson et al. 2005) where the primary focus remains studying $\delta^{13}C$ in organic tissues. On the other hand breath testing using artificially enriched ^{13}C-tracers has been most influenced by clinical research—namely the study of metabolic diseases and exercise physiology.

Comparative physiologists often seek to understand both how animals interact with their environments, and thus how variation in isotope signatures in their environment are reflected in incorporated carbon, as well as their internal metabolic processes that govern how carbon isotopes are routed through the body. In addressing both sets of questions they must be able to bridge the knowledge base developed by both ecologists and clinicians. While either approach can potentially be used to investigate a given question, there are some important distinctions between them. In the next section, we discuss the background and application of each methodology.

Natural abundance

Breath testing studies using natural abundance levels of ^{13}C rely on the relatively small differences in $\delta^{13}C$ that result from isotopic fractionation. Fractionation refers to the physical or biochemical processes that result in the differential distribution of heavy and light isotopes of a given element (e.g., ^{13}C and ^{12}C). Because lighter isotopes tend to react faster in forward-moving, kinetic reactions (Farquhar et al. 1989; Fry 2006; O'Leary 1988; O'Leary et al. 1992) the pool of chemical reactants tends to become isotopically heavier than

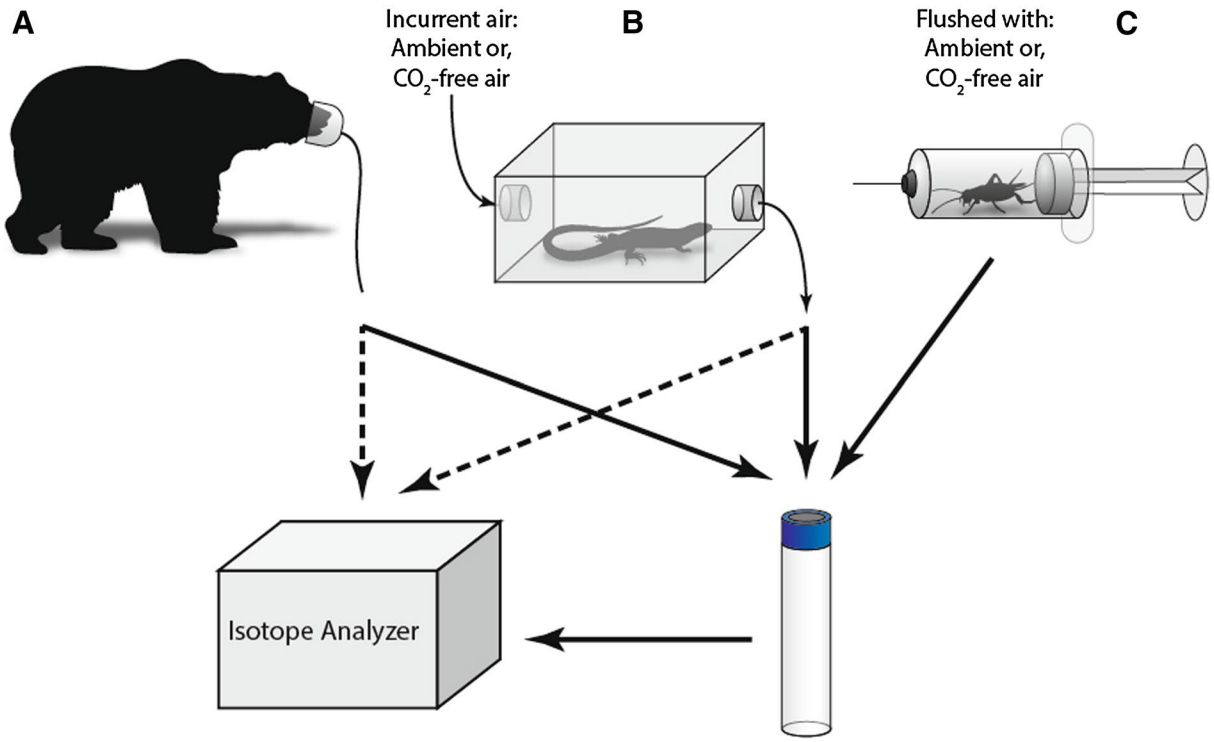

Fig. 1 Common approaches used for collecting the breath of animals for ^{13}C analyses. **a** Breath can be collected directly at the nostril or subsampled using a mask. In this case, the inclusion of ambient CO_2 is typically unavoidable and the error introduced by mixing of CO_2 from the air and the organism can be minimized either by collection of sufficiently exhalation-enriched CO_2 concentration or use of artificially-enriched tracers or corrected for by measurement of the isotopic signature of ambient CO_2 and relative CO_2 concentrations from each source. **b** Excurrent gas from a flow-through metabolic chamber can be captured for analysis. In this case, incurrent air can be made CO_2-free by use of chemical scrubbers or purified gasses. If ambient air is drawn into the chamber, the error introduced by the mixing of environmental CO_2 can be dealt with as in **a**. **c** Small animals can be placed inside an appropriately sized syringe or sealed respirometry chamber to collect the breath. The syringe could be flushed with CO_2-free air prior to sampling. In all three examples above, sampled gases can be stored in evacuated glass containers for up to several weeks prior to analysis (*solid arrows*). When a 'real-time' isotope analyzer is used (Table 1) air samples from examples **a** and **b** above can be fed directly into the analyzer for analyzer for immediate analysis (*dashed arrows*). These are only examples and these different methods are not mutually exclusive from one another

a pool of products. In other words, the $\delta^{13}C$ in products is lower than the $\delta^{13}C$ in the reactants. In exchange reactions, where both forward and reverse reactions are possible, the heavy isotopes tend to accumulate in the molecular species where the bonds are strongest (Fry 2006; Martinez del Rio and Wolf 2005; O'Leary et al. 1992). In living tissues both forward-moving and exchange reactions are continually occurring leading to infinitely complex fractionation patterns (Bier 1997; Doronin et al. 2012; Hayes 2001; Martinez del Rio and Wolf 2005). Fortunately, there are two overarching drivers of fractionation whose effects can be observed among animals—and both of them can be exploited for purposes of ^{13}C-breath testing.

C3 vs. C4 plants

Plants with photosynthetic pathways that are characterized as C3 and C4 have distinct $\delta^{13}C$ values as the result of fractionation of atmospheric $^{13}CO_2$ as it is captured and incorporated into the growing plant tissues (reviewed in Ambrose and DeNiro 1986; Coplen et al. 2002; Farquhar et al. 1989; O'Leary et al. 1992; Park and Epstein 1961). In general, C4 plants have $\delta^{13}C$ values that are ~8–16 ‰ higher than C3 plants (Fig. 2). The $\delta^{13}C$ in CAM plants is more variable and usually resembles those of C4 plants (Wolf and Hatch 2011), but may also fall within the ranges reported for C3 plants depending on the environmental conditions (Pate 2001). For the most part this variation in $\delta^{13}C$ values of primary producers is maintained across trophic levels such that the $\delta^{13}C$ of the tissues and of breath in animals, and is correlated with those of their respective diets.

Interestingly, these physiological differences in C3 and C4 plants are also ultimately responsible for the naturally occurring differences in the $\delta^{13}C$ in the breath of different human populations. For example because Europeans tend to consume sugar derived from sugar beets (i.e., a C3 plant), rather than corn or sugar cane (i.e., C4 plants) like

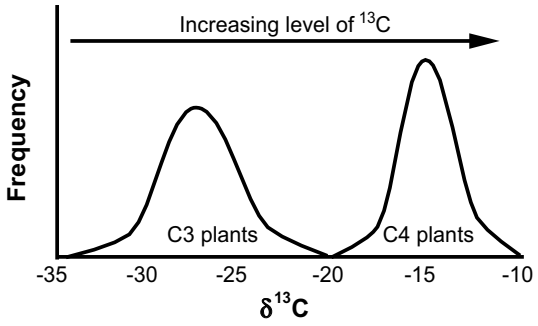

Fig. 2 Frequency distribution of the $\delta^{13}C$ in the bulk tissues of hundreds of plant species. Redrawn from data presented in (O'Leary 1988). The $\delta^{13}C$ in the tissues of CAM plants are more variable and can resemble those of C3 or C4 plants depending on the condition (Pate 2001)

their American or African counterparts, their breath tends to exhibit a lower δ^{13}C (Gautier et al. 1993; Massicotte et al. 1993; Schoeller et al. 1980; Tanis et al. 2000; Wagenmakers et al. 1993). Although it has not been employed this difference in the breath could be exploited and serve as a minimally invasive screening technique to complement existing customs and border enforcement practices.

Lipids vs. nonlipids

Within a given organism (e.g., plant or animal) the lipid molecules tend to have a δ^{13}C that is ~2–8 ‰ lower than non-lipid molecules. There are several biochemical branch points during lipid synthesis where discrimination against ^{13}C-containing molecules may occur (Hayes 2001). For example pyruvate containing ^{13}C is slightly less likely to be converted into acetyl-CoA by the pyruvate dehydrogenase complex (DeNiro and Epstein 1977; Ruess et al. 2005). Moreover, ^{13}C-acetyl CoA is slightly less likely to become integrated into growing fatty acid chains than ^{12}C-acetyl CoA (Blair et al. 1985; Des Rosiers et al. 1991). There is also evidence that ^{13}C-containing fatty acids undergo discrimination during esterification and deesterification with glycerol molecules (reviewed in Welch et al. 2015).

In studies of the δ^{13}C in bulk tissues of plants and animals these differences between lipid and nonlipid components have been considered an unwanted source of variability (e.g., Barnes et al. 2007; Focken and Becker 1998; Hoffman and Sutton 2010; Hussey et al. 2010; Logan et al. 2008; Oppel et al. 2010; Post et al. 2007) that must be chemically or mathematically factored out. In contrast, ^{13}C-breath testing studies have been able to exploit this naturally occurring difference. For example, overweight patients on calorie-restricted diets tend to burn a greater proportion of endogenous lipid and therefore exhibit lower δ^{13}C in their breath. Periodic, minimally invasive monitoring of their breath can be tracked ensure they are complying with their prescribed diets over extended periods (Whigham et al. 2014). Other examples of how the difference in δ^{13}C between lipids and nonlipid has been exploited in comparative physiology are discussed below.

Artificial enrichment

The naturally occurring differences in δ^{13}C of C3 versus C4 plants or lipid *versus* non-lipid molecules are ubiquitous—and free to users. But there are two important advantages of using artificially enriched ^{13}C-tracers for breath testing. The first is that artificially enriched ^{13}C-tracers enable researchers to study the oxidative fates of a greater variety of specific types of molecules rather than the hundreds of compounds found in a typical plant or animal tissue. This lack of molecular specificity has been referred to as the 'scrambled-egg premise' (sensu Van Der Merwe 1982). The limitation of molecular specificity inherent within the scrambled egg premise can be minimized, in part, by using laboratory synthesized large molecules such as ^{13}C-algal or ^{13}C-milk proteins. However insight into the oxidative fates of the constituent amino acids of these proteins is precluded because they exhibit their own unique δ^{13}C (Macko et al. 1983; Newsome et al. 2014). Some of the early ^{13}C-breath testing studies in humans (e.g., Lacroix et al. 1973) and later in comparative models (e.g., Voigt and Speakman 2007; Welch et al. 2006; Welch and Suarez 2007) elegantly circumvented the scrambled egg premise by using purified fractions (e.g., glucose or fructose) derived from C3 and C4 plants. Unfortunately, this approach is not practical for other plant fractions (e.g., oils or proteins) that have heterogenous compositions.

The second advantage is that artificially enriched tracers can be used to generate larger changes in the δ^{13}C in the breath. For example the breath of a 15 g zebra finch ingesting 5 mg of ^{13}C-1-glucose will reach values of >300 ‰ within 15 min and remain >50 ‰ over the subsequent 2 h (MDM personal observation). This increased isotopic signal (compared to ≤16 ‰ employing natural C3 vs. C4-derived variation) makes it easier to identify and characterize relatively small changes in oxidation (Gay et al. 1994; McCue 2011) or to do so using less precise (i.e., less expensive) analytical equipment.

Each year new artificially enriched ^{13}C-molecules become commercially available from producers such as Cambridge Isotope Laboratories (CIL; Tewksbury, MA) and ISOTEC (Sigma–Aldrich). CIL presently produces over 10,000 different ^{13}C-labelled molecules (R. Trolard, personal communication). The large combination of ^{13}C-labelled molecules stems in part from the fact that a small molecule such as glucose with six carbons could be ^{13}C-labelled in up to 63 different combinations. Although

each carbon atom in a glucose molecule could potentially undergo a different biochemical fate (Chown et al. 2012; Coggan 1999; Murphy 2006; Obeid et al. 2000), knowledge of this level of intermediary metabolism is not practical for breath testing involving animals. As such, most breath testing studies employing artificial enrichment use glucose molecules where all of the carbons are uniformly labelled with ^{13}C (i.e., U-^{13}C-glucose) or a less expensive option where only the number one carbon atom is labelled (e.g., 1-^{13}C-glucose). Below we discuss other 'general-purpose' tracers for fatty acids and amino acids.

When purified, low molecular weight ^{13}C-tracers (e.g., monosaccharides, amino acids, or fatty acids) are administered along with a normal, mixed diet the meal is said to be extrinsically labelled. In other words, the ^{13}C-tracer is not biochemically integrated into the meal. However, researchers have combined ^{13}C-tracers with both plants and animals to generate foods that are intrinsically labelled to study oxidative kinetics in a more realistic way (Boirie et al. 1996; Ghoos and Beaufrere 1998). Cows fed with ^{13}C-amino acids will produce ^{13}C-enriched milk that can be separated into whey and casein proteins (Boirie et al. 1995). Chickens and quail fed ^{13}C-proteins or amino acids (Berthold et al. 1991; Bujko et al. 2007; Evenepoel et al. 1997; Fromentin et al. 2012; McCue et al. 2013a) will create ^{13}C-ovalbumin. These labelled eggs and milk can even be converted into other foods like pancakes (Bammens et al. 2004; Geboes et al. 2004). Plant tissues can also become intrinsically labelled by growing them in an engineered atmosphere, directly injecting ^{13}C-labelled molecules into the stems, or adding ^{13}C-labelled molecules to hydroponic systems (reviewed in Grusak 1997). One study describes how ^{13}C-labelled pasta was produced from wheat raised in a $^{13}CO_2$ atmosphere (Folch et al. 2001).

Sample "contamination" with environmental CO_2

The environment around air breathing animals is composed of, on average, 0.04 % CO_2. In many cases, it is impossible (Fig. 1a) or impractical (e.g., flow-through sampling from a respirometry chamber in the field, or employing high flow rates; Fig. 1b) to exclude this ambient CO_2 from the breath sample to be analyzed. Chemically-scrubbed or purified CO_2-free air can be used to flush a small, sealed collection chamber (Fig. 1c) or can be used to provide the incurrent flow for a flow-through respirometry chamber (Fig. 1b). Doing so avoids the issue of contamination altogether.

The mixing of ambient environmental CO_2 with that from the breath of a study organism has the potential to obscure accurate measurement of $\delta^{13}C$ from the breath CO_2 derived from the oxidation of a tracer substrate. This is particularly true when the concentration of breath CO_2 in the gas sample to be analyzed is low (e.g., <1 %) and/or when the isotopic signature of the tracer molecule is relatively similar to other potential oxidative substrates and ambient CO_2 (i.e., when relying on naturally enriched tracers). Problematic contamination could also arise from the proximity of air-breathing researchers or CO_2-emitting machines (e.g., running internal combustion engines or flames), though such contamination can be avoided by sampling in well-ventilated areas and by excluding any unnecessary CO_2-emmitters (including accessory researchers).

Fortunately, even the potential error arising from contamination with ubiquitous ambient CO_2 can be minimized in several ways. Even relatively small respiring organisms breathing and rebreathing air within even an unsealed small container or mask can quickly raise the concentration of CO_2 to above 2 %. At this concentration, the relative amounts of organism-derived CO_2 dwarfs that of ambient CO_2 by roughly 50 times. At these relative levels, the error introduced by ambient CO_2 is less than the precision of many isotope analyzers. Similarly, when artificially enriched tracers are used, the magnitude of the difference between the isotopic signature of CO_2 derived from oxidation of the tracer and ambient CO_2 or other oxidative substrates is so great that potential error from contamination is negligible.

It is also possible to mathematically correct for ambient CO_2 contamination. If the CO_2 concentration and isotopic signature are known for both the breath + ambient air sample as well as the ambient sample alone (sampling of well-mixed air in the absence of the study animal), a simple two part mixing model can be applied to calculate the signature of the pure breath CO_2 (Welch et al. 2006; Welch and Suarez 2007; Welch et al. 2008). Lastly, even if the concentration and signature of ambient CO_2 are not measured, the signature of 'pure' breath CO_2 can be estimated by plotting the $\delta^{13}C$ of the breath + ambient air sample against the reciprocal of the total CO_2 concentration in this sample [i.e., Keeling plot (Carleton et al. 2004)]. Extrapolation of a linear regression of repeated samples (varying in total CO_2 concentration) can be used to predict 'pure' breath $\delta^{13}C$ as the value of the *y*-intercept [i.e., the point at which the total CO_2 concentration is infinitely high as a result of concentration of breath CO_2 within the sample (Carleton et al. 2004)].

Applications

Breath testing studies can also be categorized according to the source of the nutrient of interest (e.g., endogenous versus exogenous) and further according to the time window of interest (e.g., acute versus chronic). In general, studies examining how exogenous nutrients affect the breath may be referred to as 'diet switching' whereas studies examining the relationship between the oxidation of endogenous

nutrients and the breath may be referred to as 'fuel switching'. Below we summarize how these breath testing approaches can provide different insights depending on the research interest.

Diet switching

Diet switching occurs when an animal that has previously been consuming a diet with a given isotopic composition is presented with another diet with a distinct $\delta^{13}C$. When combined with breath testing, diet switching studies can be used to determine how soon after ingestion exogenous nutrients are used as metabolic fuels and how long after feeding they continue to provide energy.

The most common form of acute diet switching used in breath testing studies involves animals raised on a diet derived from C3 plants that are switched to a meal derived from C4 plants (Fig. 3a) causing the breath to become isotopically heavier. Diet switching can also involve changing an animal's diet from C4 plants to C3 plants in which case the breath becomes isotopically lighter. In either case, the $\delta^{13}C$ in the breath can be incorporated into the following mixing model

$$\delta^{13}C_{breath} = \delta^{13}C_{meal}(x) + \delta^{13}C_{tissues}(1 - x) \quad (4)$$

that accounts for the $\delta^{13}C$ in the meal and the $\delta^{13}C$ in the tissues to estimate the relative reliance on dietary nutrients (x) to total nutrient oxidation during the postprandial period (e.g., Amitai et al. 2010; Carleton et al. 2004; Voigt and Speakman 2007; Welch et al. 2006, 2008; Welch and Suarez 2007). Changes in the $\delta^{13}C$ can also be used to determine the time at which a fed animal returns to a post-absorptive state (Fig. 3a).

If the diet switch is chronic then the $\delta^{13}C$ of endogenous fuel stores, and thus the $\delta^{13}C$ of the breath, derived from oxidation of either exogenous or endogenous fuels, will gradually change until it approaches the $\delta^{13}C$ of the new diet (Fig. 3b). This can take days or months or longer, chiefly depending on the size of the animal and the carbon turnover rate in the tissues (Hobson and Yohannes 2007; MacAvoy and Arneson 2006; Martinez del Rio and Carleton 2012; Sponheimer et al. 2006). We are not aware of any diet switching studies that have actually documented the time required for all of the tissues and the breath to come into isotopic equilibrium with a new diet, but these times can be estimated (e.g., Kurle et al. 2014; Sponheimer et al. 2006).

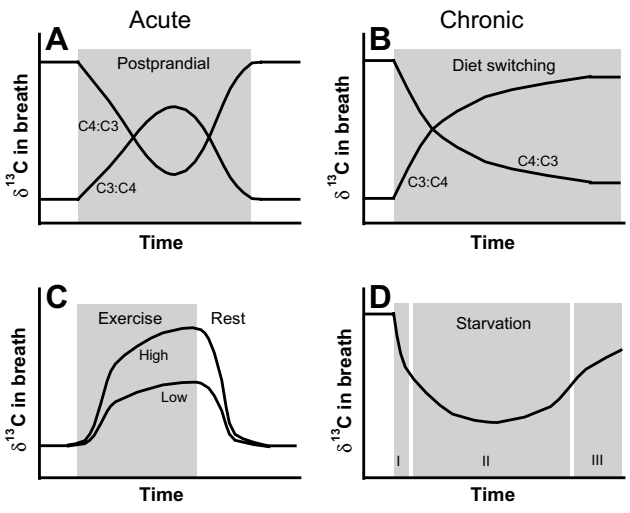

Fig. 3 Generalized outcomes of breath testing at natural abundance levels under several conditions. **a** Short-term diet switches from either a C3 to C4 diet or a C4 to C3 diet. **b** Long-term diet switches from either a C3 to C4 diet or a C4 to C3 diet. **c** Exercise at high or low intensity. **d** During starvation across fasting phases I, II, and III [sensu (Castellini and Rea 1992)]

Fuel switching

Proteins, lipids, and carbohydrates comprise the three major classes of metabolic fuels. Animals oxidize a finite amount of each of these nutrients at any given time, but the relative reliance on these three fuels varies widely—among different animals and across different physiological states. The shifting reliance on these three fuels, a phenomenon we refer to here as 'fuel switching' has traditionally been characterized using the ratio of an animal's rate of CO_2 production to its rate of O_2 consumption—a measure called respiratory exchange ratio (RER). But, the interpretation of fluctuations in RER to quantify changes in fuel use can be problematic (Goedecke et al. 2000; Kleiber 1975; Lighton and Halsey 2011; Mahon and Timmons 2014; Malte et al. 2015; Venables et al. 2005; Walsberg and Wolf 1995). While RER can be used to infer relative reliance on one class of fuels versus another (particularly if the contribution of one of the three fuel classes, like proteins, can be assumed to be negligible), it does not easily permit discrimination among different types of fuels within a class.

When it is assumed that proteins account for a negligible proportion of overall fuel oxidized, such as during exercise in well-fed mammals, an RER ~1.0 indicates reliance predominantly or exclusively on carbohydrate oxidation, an RER ~0.7 indicates reliance on lipids, and values in between indicate some mixture of the two. However, RER often cannot reliably reveal what proportion of each fuel class in use, carbohydrates or lipids, is derived from endogenous versus exogenous stores, nor assign, with confidence, the exact identity of component fuel class compounds. Even when the change in RER following ingestion of a particular fuel, such as glucose, is large, such a change may represent both increasing reliance on the newly ingested sugar as well as a correlated increase in

endogenous carbohydrate mobilization and oxidation. Fortunately, breath testing can provide a complementary tool with which to document changes in the mixtures of metabolic fuels, particularly to discriminate among fuel classes.

Exercise

It is often assumed that well-nourished animals oxidize a negligible amount of their endogenous proteins (Gautier et al. 1993; Sonko et al. 2005). This assumption is particularly evident in some areas of research (e.g., exercise physiology, weight loss studies) where the oxidative fates of lipids and carbohydrates are the central focus. But recent studies of birds, rodents, and insects suggest this assumption may not be valid and that rates of protein oxidation in nourished, postabsorptive animals can be quite high (McCue et al. 2013b, 2015c; McCue and Pollock 2013). Nevertheless, if we accept the assumption that lipids and carbohydrates are the most important fuels (or at least that the rates of protein oxidation are constant) then breath testing can be used to qualitatively document fuel switching during exercise. Breath testing in humans has shown that the $\delta^{13}C$ increases during acute exercise as the body decreases its relative reliance on the isotopically lighter endogenous lipids and increases its reliance on carbohydrate oxidation (Gay et al. 1994; Jandrain et al. 1993; Romijn et al. 1992; Wolfe et al. 1984). A recent study using larger sample sizes and multiple treatment levels showed that the increase in the $\delta^{13}C$ was directly proportional to exercise intensity (McCue et al. 2015b) (Fig. 3c) reflecting the 'crossover-concept' well known to exercise physiologists (Brooks and Mercier 1994; Venables et al. 2005).

Fasting

Animals differ widely in their ability to tolerate periods of food limitation, but if the $\delta^{13}C$ in their breath is tracked over these periods then two general patterns are usually seen. First, the $\delta^{13}C$ in the breath initially decreases as a result of an increased reliance on oxidation of endogenous lipids (Fig. 3d). Second, as fasting progresses, the $\delta^{13}C$ in the breath gradually increases as the relative reliance on protein oxidation increases. The timing of these transitions may provide insight into the progression of sequential phases of fasting and starvation (Khalilieh et al. 2012). Below we provide specific examples from breath testing in fasting animals.

Tracers are not created equal

Breath testing has shown us that different classes of nutrients, and even different monomers within each class may have unique oxidative kinetics. This variation is problematic in some cases, but it also offers researchers an opportunity. The problem arises from the fact that it is difficult to directly compare and interpret the results of breathing testing studies that use different molecules to represent a given class of nutrients (e.g., glucose versus fructose). On the other hand breath testing allows researchers to explore how minute differences in chemical structure influence the oxidative disposal of closely related molecules. In the following section we outline some of these differences and review how experimental factors such as nutritional history can alter how and when a given nutrient is oxidized.

Sugars and starches

Exercise physiologists using breath testing have repeatedly shown that humans tend to oxidize dietary glucose to a greater extent than fructose (Jandrain et al. 1993; Massicotte et al. 1986, 1989). Nutritionists have shown that dietary history affects monosaccharide oxidation. In particular acclimation to higher carbohydrate diets caused humans to oxidize dietary glucose to a greater extent than those acclimated to low-carbohydrate diets (Koutsari and Sidossis 2003). Molecule size can also affect tracer oxidation. One study shows that soluble sugars are oxidized more readily than starches (Saris et al. 1993) or other glucose polymers (Massicotte et al. 1989). Even within starches there is variation in oxidative disposal. Another study reports that humans oxidized extruded starch more rapidly than crystalline starch, and that waxy starch was oxidized more rapidly than high amylose starch (Hiele et al. 1990).

Fatty acids and lipids

The body oxidizes various classes of lipids and fatty acids at different rates. Breath testing in humans shows that dietary oleic acid is oxidized more rapidly than stearic acid (Jones et al. 1985) and linoleic acid (McCloy et al. 2004). A study of sparrows reports that oleic acid was oxidized faster than stearic acid, but not as fast as palmitic acid (McCue et al. 2010b).

As with carbohydrates, dietary history can affect the oxidative kinetics of fatty acids. Women oxidized more long chain fatty acids when they were acclimated to diets rich in medium-chain fatty acids (Papamandjaris et al. 2000). Rats raised on diets low in $n-3$ fatty acids oxidized less docosahexaenoic acid (DHA) than controls (Waki et al. 2004). But not all studies report a link between dietary history and oxidation kinetics; humans acclimated to diets rich in either monounsaturated or saturated fatty acids did not differ in the oxidative disposal of dietary oleic acid (Cooper et al. 2010).

The oxidation of triacylglycerols can also vary. For example children oxidized trioctanoin more rapidly than

triolein (Koletzko et al. 1998). Quail and geckos oxidized palmitic acid far more rapidly than tripalmitin (McCue and Cardentey 2014). Finally, the route of ^{13}C-tracer administration affects the oxidative fates of lipids, and humans oxidized oral doses of triolein at much greater rates than identical intravenous doses (Metges and Wolfram 1991).

Amino acids and proteins

Amino acids are oxidized differently depending on whether or not they are essential. For example sparrows oxidized dietary glycine (nonessential) far more extensively than leucine (essential) (McCue et al. 2010a). Molecular complexity is also a factor influencing the kinetics of oxidation of component amino acids. Humans and rats fed extrinsically labelled ^{13}C-leucine oxidized it faster than when they were fed proteins intrinsically labelled with ^{13}C-leucine (Bujko et al. 2007; Metges et al. 2000). Similar results were found in quail and geckos fed intrinsically and extrinsically ^{13}C-labelled proteins (McCue and Cardentey 2014).

Breath testing has shown that nutritional history affects the oxidative disposal of amino acids. For example chickens raised on a low protein diet oxidized less dietary leucine than controls (Swennen et al. 2007), and zebra finches recovering from prolonged fasting oxidized less dietary leucine than nourished birds (McCue et al. 2011). In both cases the birds in the experimental groups were believed to be reserving dietary amino acids to support needed protein synthesis.

Oxidative kinetics also differ among different types of proteins. For example humans oxidize soy proteins more rapidly than milk proteins (Bos et al. 2003). Even within milk the whey proteins have been shown to be oxidized faster than the casein (Boirie et al. 1995; Bos et al. 1999), leading to difficulty in modeling the oxidative kinetics of complex foods using a single-pool approach (see next section).

Modeling carbon turnover kinetics

For simplicity the incorporation of dietary ^{13}C atoms into the body or a particular tissue is usually described using a 1-compartment model (e.g., Bearhop et al. 2002). In this case, the isotopic value of the animal or tissue asymptotically approaches that of the diet such that it can be mathematically modeled using an equation that contains a single exponent characterizing the 'incorporation rate' of carbon into the target pool (Martinez del Rio and Carleton 2012). However, this assumption overlooks the integrative nature of different carbon pools within an animal (Ayliffe et al. 2004; Bier 1997; Martinez del Rio and Carleton 2012; Warne et al. 2010; Wolfe and Chinkes 2005).

In some cases, the mathematical model that best approximates observed changes in the ^{13}C signature of the animal or target tissue is not a simple exponential function and instead incorporates two (or more) parameters describing 'incorporation rates' of carbon into two (or more) theoretical compartments (Martinez del Rio and Carleton 2012). Using a diet-switching approach, Carleton and Martinez del Rio (2008) compared 1- and 2-compartment models describing the integration of carbon into sparrow tissues. While 1-compartment models best approximated carbon integration kinetics into the breath and splanchnic tissue pools, 2-compartment models better described integration kinetics into plasma and metabolically active tissues such as intestine and liver (Carleton et al. 2008). Studies of carbon turnover in the zebra finch (Bauchinger and McWilliams 2009) and of washout kinetics in the egg proteins in quail (McCue et al. 2013a) similarly found that 2-compartment models best described turnover in some pools.

Modeling the appearance of δ^{13}C in the breath using a 1-compartment approach is fraught with issues similar to those in the tissues. Even meals that are intrinsically ^{13}C-labelled may contain complex nutrient mixtures containing components that are differentially absorbed by the gut, routed to organs, and catabolized by cells. Breath testing comparing orally and intravenously dosed proteins shows that first-pass splanchnic oxidation accounts for ~20–30 % of the oxidation of dietary proteins (Crenn et al. 2000; Istfan et al. 1988; Kriengsinyos et al. 2002; Metges et al. 2000). The fact that proteins are broken down at different rates and that constituent amino acids are also oxidized at different rates (Crenn et al. 2000; Daenzer et al. 2001; Deglaire et al. 2009; Stoll et al. 1998) can explain why the casein and whey components of protein in milk experience dramatically different mobilization and oxidation kinetics from one another (Boirie et al. 1997; Bos et al. 1999; Ghoos and Beaufrere 1998). Second, because incorporation of labelled carbon into some endogenous pools is better approximated by 2-compartment models, such complexity is likely apparent in the rates at which carbon leaves these pools. Thus, changes in the δ^{13}C of breath that track the oxidation of endogenous nutrients from these pools may not be well approximated by 1-compartment modeling approaches, and this uncertainty should be considered when interpreting such data.

An additional technical concern relates to the loss of ^{13}C through tricarboxylic acid cycle intermediates and via loss of CO_2 through incorporation of plasma bicarbonate into non-respiratory carbon pools such as urea (Elia et al. 1988). In both cases, these losses would, if unaccounted for, lead to an underestimation of the proportion of labelled carbon that has been oxidized. Various studies have combined administration or infusion of labelled carbon substrates (e.g., glucose, amino acids, lipids) with labelled carbon

intermediates (e.g., acetate or bicarbonate) to estimate carbon losses through each route (Burelle et al. 2006; Sidossis et al. 1995; Trimmer et al. 2001). Estimates vary, however, and are likely influenced by ontogeny, nutritional history, and activity states (Hambly and Voigt 2011; Leslie et al. 2006; Moehn et al. 2004).

Rest and digest states

Clinical applications

Breath testing has been used for decades by clinicians to identify disease states related to gastrointestinal function (see detailed reviews in Braden 2010; Evenepoel et al. 2000; Fischer and Wetzel 2002; Ghoos and Beaufrere 1998; Koletzko et al. 1998; Parra and Martinez 2006; Rating and Langhans 1997). In practice the patient consumes a specific ^{13}C-tracer and the timing and magnitude of changes in the δ^{13}C of the breath are monitored over the following 1-4 h. The most commonly employed of these is the ^{13}C-urea test for the presence of *Helicobacter pylori* which is the leading cause of gastric and duodenal ulcers. Normally the body does not oxidize urea and if ^{13}C is recovered in the breath following ^{13}C-urea administration the patient is infected (Fig. 4a). Similarly, a conclusion of overgrowth of bacteria in the small intestine is reached if δ^{13}C in the breath becomes elevated after ingestion of ^{13}C-xylose or ^{13}C-sorbitol (Fig. 4b).

Not all of these tests involve bacteria. Delayed gastric emptying can be detected using ^{13}C-acetate (Fig. 4c). Detection of fat malabsorption and pancreatic dysfunction usually involve administration of ^{13}C-labelled triacylglycerol (e.g., triolein or tripalmitin). In practice a diminished appearance of ^{13}C in the breath after ingesting these tracers could indicate failure to secrete sufficient levels of bile or pancreatic lipases thereby causing fat malabsorption (Fig. 4d). Failure of the pancreas to secrete peptidases can be detected using a dipeptide that is not digested by gastric protease but is only broken down in the duodenum (Kohno et al. 2007). Other specific disorders of liver function may be identified using various ^{13}C-labelled molecules (e.g., fructose, galactose, caffeine, erythromycin, phenylalanine, and tyrosine).

To date, very few of these clinically important tracers have been used to investigate questions in comparative physiology, but these tools could provide a fertile area for future research. For example, the ^{13}C-urea test described above has been used to accurately diagnose *H. pylori* infections in cheetahs (Chatfield et al. 2004), but it could be adapted to determine whether this waste product that is toxic to many animals is a viable source of metabolic energy in other animals such as hibernating bears (e.g., Harlow 2012). Other new applications for breath testing in comparative physiology are outlined in the final section of this review.

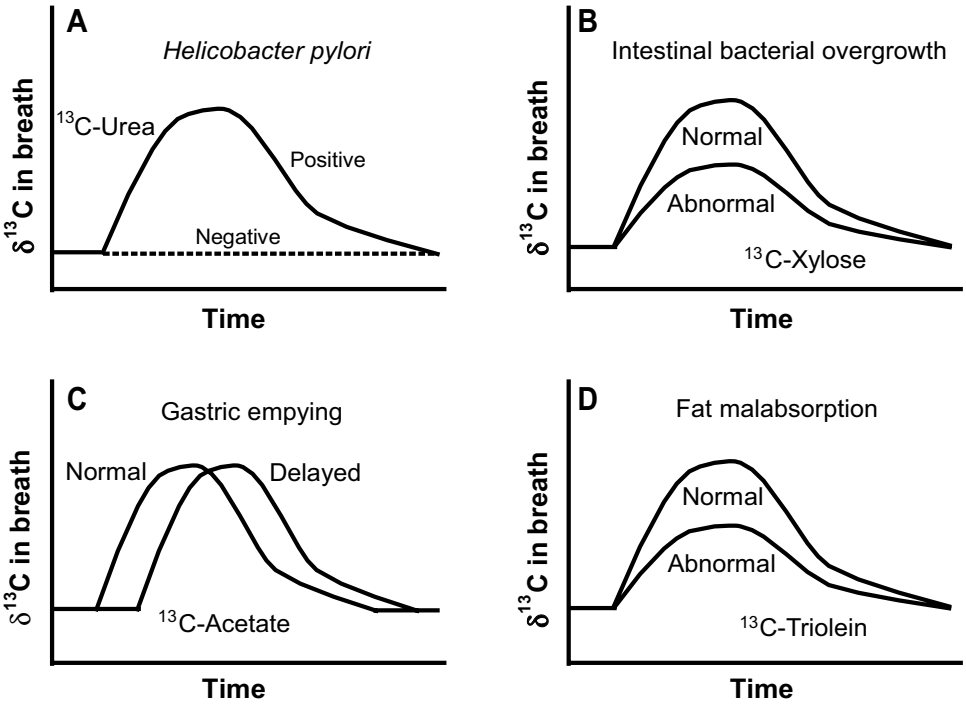

Fig. 4 Generalized outcomes of breath tests used for diagnosing human disease states

Comparative models

Breath testing has been used to study the effects of thermal acclimation in animals. Siberian hamsters were acclimated to 10 °C for two weeks to induce the upregulation of brown adipose tissue (BAT) and then fed meals spiked with ^{13}C-labelled glucose, leucine, or palmitic acid tracers before an acute cold challenge (i.e., 3 h of exposure to 2 °C). While the cold-acclimated hamsters oxidized the leucine and glucose at the same rate as their control counterparts, they oxidized 30 % more palmitic acid, suggesting that they were powering some of the nonshivering thermogenesis in BAT using recently ingested fatty acids (McCue et al. 2014). Breath testing has also been used to study age and developmental differences among animals. For example ^{13}C-glucose tracers combined with breath testing revealed that older chickens oxidized incoming glucose more extensively than younger hens permitting researchers to interpret their results in the context of hormonal differences (Buyse et al. 2004).

Pythons have become model organisms in which to study the physiological responses to digesting large meals in part because they demonstrate exceptionally large and protracted changes in morphology and metabolic rate during digestion (Secor and Diamond 1998). Breath testing on pythons digesting feeder mice raised on C3 or C4 diets revealed that a substantial amount of the heat increment of feeding, also known as specific dynamic action, was fueled by the oxidation of the exogenous nutrients in the meal (Starck et al. 2004; Waas et al. 2010). In a recent follow-up study feeder mice were raised on ^{13}C-leucine or ^{13}C-palmitic acid tracers to intrinsically label the proteins and lipids, respectively (McCue et al. 2015a). When these mice were fed to pythons breath testing showed that the snakes were able to partition the different classes of nutrients in their meal (Fig. 5a). In particular the snakes only oxidized the proteins in the meal during the first half of digestion. Moreover, while their reliance on exogenous lipid oxidation during digestion was minimal they continued to oxidize the lipids in the mouse meals long into the postabsorptive period.

Fasting and starvation states

Most animals do not continually process food, but rather eat in discrete events. These regular feeding cycles cause the body to switch between an anabolic state when it is in positive energy balance and a catabolic state when it is in negative energy balance. Breath testing using either natural abundance or artificially enriched approaches can provide insights into the specific source of metabolic energy used during these different periods.

Natural abundance

Stored glycogen provides a relatively short term source of metabolic energy [reviewed in Hall (2012)]. Researchers used a C3 to C4 diet switch in humans over 24 h to isotopically enrich glycogen stores. This short diet switching period was chosen to avoid the ^{13}C-enrichement in the lipid

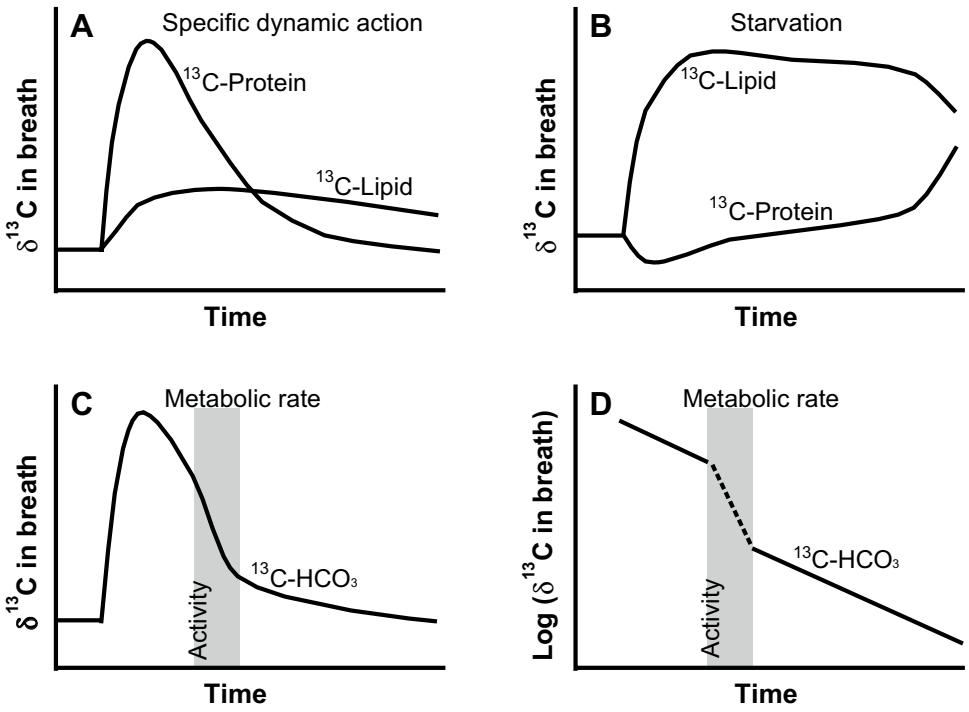

Fig. 5 Generalized outcomes of breath testing experiments investigating digestion, fasting, and locomotor activity. See text for detailed explanations

and protein pools in the body associated with longer periods of ^{13}C-glucose ingestion [e.g., (McCue et al. 2013b; Tanis et al. 2003)]. They found that the δ^{13}C in the breath peaked between 12 and 24 h into fasting during the period when endogenous glycogen was fueling energy demands (Tanis et al. 1998, 2000). Unfortunately, they were unable to distinguish between muscle and hepatic glycogen stores (Tanis et al. 2003).

During longer periods of fasting the glycogen stores become exhausted and animals will increase their relative reliance on endogenous lipids (McCue 2010) which are naturally more depleted in ^{13}C than carbohydrates and proteins (see above). This fasting-induced fuel switch is responsible for a 2 ‰ decrease in the δ^{13}C of the breath in mice fasted for 7 h (Perkins and Speakman 2001), a 5 ‰ decrease in pigeons fasted for 24 h (Hatch et al. 2002), and 2.5 ‰ reduction in cockroaches starved for 20 days (Miller et al. 1985). The $\delta^{13}C$ in the breath of fasting rats reached a nadir at 2 days after which the values began to increase over the subsequent week as they increased reliance on protein stores (Guzman et al. 2015). Given that the δ^{13}C of the body lipids significantly differs from that of the lean tissues in the body a mixing model similar to the one described in Eq. 4 may be used to estimate the extent to which endogenous lipids contribute to the overall energy demand in fasting animals at any time point.

Artificial enrichment

Artificial ^{13}C-tracers can also be used for breath testing in fasting animals. It is a labor-intensive approach, but it offers stronger isotopic signals and increased nutrient specificity. Two populations of Japanese quail raised from hatching on ^{13}C-amino acids or ^{13}C-fatty acids to enrich their endogenous proteins or lipids, respectively, exhibited a rapid yet sustained increase in their relative reliance on lipid oxidation during fasting (McCue et al. 2013b). Interestingly, breath testing revealed that over several days of continued fasting their reliance on protein oxidation began to show strong circadian fluctuations. This 24-h pattern was not seen in a similar experiment using fasted mice (McCue and Pollock 2013) suggesting that vertebrates exhibit differing strategies of rationing endogenous fuels during food limitation.

Because of their enormous ecological diversity and that fact that they can be starved to lethal endpoints without special permitting insects have recently become the subject of breath testing studies of starvation. Although insects apparently exhibit a wide variety of different starvation strategies (McCue et al. 2015c) many species show the general trend whereby they rapidly increase lipid oxidation and decrease normal rates of protein oxidation at the onset of starvation (Fig. 5b). During the final phase of starvation a decrease in lipid oxidation can be seen accompanied by a rapid spike in protein oxidation that precedes death.

Active states

During exercise, it is advantageous to be able to rapidly oxidize exogenous nutrients in order to spare endogenous stores. Breath testing has been used to identify which doses (Peronnet et al. 1993; Smith et al. 2010) or mixtures of nutrients (usually sugars) (Burelle et al. 2006; Jentjens et al. 2004a, b; Smith et al. 2010) are most readily oxidized during acute bouts of exercise. Breath testing has also been used to demonstrate that the addition of liquids facilitates gastric emptying thereby increasing the bioavailability of nutrients ingested during exercise (Mudambo et al. 1997).

Chronic exercise training causes a cascade of physiological responses including improving the body's ability to oxidize endogenous lipids (Phillips et al. 1996). Such changes in the ratios of metabolic fuels have historically been inferred from changes in RER, but breath testing in humans infused with ^{13}C-tracers can provide complementary insights into how exercising training affects the oxidation of particular metabolic substrates in circulation (Van Loon et al. 1999).

Unlike exogenous carbohydrates, it is commonly believed that ingested fats are generally not as readily oxidized during exercise in humans. Because variation in RER values cannot be used to discriminate the relative contribution of endogenous versus exogenous substrates within a given class (e.g., lipids or carbohydrates) breath testing methodologies have provided a critical complementary tool with which to address reliance on specific subclasses of nutrients. Breath testing studies have revealed that the availability and use of exogenous triglycerides as an oxidative fuel during exercise is dependent on both the chain length and method of administration. Such studies in humans have demonstrated that medium chain triglycerides (MCTs) are oxidized at comparatively faster rates than long chain triglycerides (LCTs) (Decombaz et al. 1983; Massicotte et al. 1992; Satabin et al. 1987). Further, MCTs are oxidized at higher rates when ingested along with carbohydrates (Jeukendrup et al. 1995). While ingestion of MCTs may be used to enhance exogenous energy availability during exercise, the human gastrointestinal tract cannot tolerate high throughput of MCTs during exercise, and overall contributions to energy demand remain limited [<10 % (Jeukendrup et al. 1995; Satabin et al. 1987)].

Flight

Humans and non-volant mammals possess a limited ability to use ingested fuels to power exercise and replenish endogenous energy stores during or between exercise

bouts (Kuipers et al. 1987; Welch Jr et al. 2015), and therefore intramuscular glycogen provides the majority of the energy to support intense locomotor activity (Brooks 1997; McClelland 2004). However, intramuscular glycogen stores, while readily accessible to the glycolytic pathway, are smaller and have lower energy density than lipid stores. Given the high mechanical and metabolic power requirement associated with flight, and the comparatively greater costs of supporting the additional weight of enhanced endogenous energy stores (compared to locomotion on the ground), it is not surprising that birds and bats can support as much as 100 % of costly flight metabolism via the oxidation of lipid stores. Indeed, using stable isotope breath tracking, several groups have shown that hummingbirds (Chen and Welch 2014; Welch et al. 2006, 2008; Welch and Chen 2014; Welch and Suarez 2007), pigeons (Hatch et al. 2002), and bats (Voigt et al. 2010) are capable of supporting flight largely or exclusively via oxidation of endogenous lipids during both short fasting periods and long distance migratory flight.

While lipids are a weight saving form of energy storage, lipogenesis from non-lipid dietary precursors can involve the loss of significant portions of ingested energy (e.g., ~15 % of the energy present in ingested glucose). Thus, there may be significant advantages to being able to directly make use of ingested fuels to fuel flight. In fact, essentially all flying vertebrates that have been examined using stable isotope breath testing following ingestion of labelled foods display impressive abilities to rapidly oxidize those exogenous fuels at comparatively high rates. Among bats, $\delta^{13}C$ values of the breath of both sanguinivores and insectivores rapidly approach the values of their meals (Voigt et al. 2008b, 2010). The protein rich diet of sanguinivorous vampire bats suggests that it is amino acids that are rapidly being mobilized and oxidized to support foraging activity. Indeed, stable isotope tracking of breath was used to show that an insectivorous bat oxidizes the protein portion of its meals to fuel ongoing flight, routing ingested lipids to endogenous stores to fuel non-foraging migratory flight (Voigt et al. 2012). Flying insects also exhibit this pattern. Breath testing on tsetse flies digesting blood meals spiked with ^{13}C-tracers shows that these animals oxidize dietary amino acids thereby sparing carbohydrates for lipid synthesis (McCue et al. 2016).

Differences in the macronutrient composition of the diet account for differences in fuel use strategy among other flying vertebrates. Stable isotope tracking of breath has shown nectarivorous hummingbirds and nectar and fruit eating bats each rapidly switch to oxidizing newly ingested simple sugars to fuel flight upon commencing feeding (Amitai et al. 2010; Chen and Welch 2014; Voigt and Speakman 2007; Welch et al. 2006, 2008; Welch and Suarez 2007). In some of these cases, researchers have shown that the animals can fuel 75–100 % of the most energetically demanding form of flight, hovering, with newly ingested sucrose, glucose, or fructose (Welch and Chen 2014). The rates at which the flight muscles of these hummingbirds and bats can take up and oxidize newly ingested sugars exceed those of any non-flying vertebrate locomotor muscles by an order of magnitude (Welch and Chen 2014) and speak to the various physiological adaptations that enhance digestive, cardiovascular, and metabolic flux in these sugar specialists, and as seen above, in birds and bats more generally.

Bicarbonate method

Apart from investigations of fuel use, injections of ^{13}C-bicarbonate followed by breath testing have been used to estimate metabolic rates in humans conducting routine activities of the course of a day (Elia et al. 1988). Unfortunately this approach may not provide estimates of CO_2 production that are more accurate than room-calorimetry methods (Lighton 2008). More recently this approach has been employed to measure the metabolic cost of short bursts of activity in mice (Speakman and Thomson 1997) and birds (Hambly et al. 2007). In practice a bolus of injected ^{13}C-bicarbonate is continually lost from the body in the breath following an exponential decay function if the animal is at rest (Fig. 5c). But during brief periods of activity the rate of $^{13}CO_2$ elimination increases proportionally to the increase in metabolic rate and this change can be used to estimate metabolic rates without the need to confine the animal in a metabolic chamber during activity (Fig. 5d). Because of the relatively short turnover of the circulating bicarbonate pool in small animals the useful measurements must be made within an hour of tracer administration.

Future directions

The studies reviewed above underscore the myriad of applications for ^{13}C-breath testing and demonstrate that they can be used in virtually any air-breathing animal. Those examples pave the way for breath testing to be used to address new questions in comparative physiology as well as provide clearer insight into classical questions about animal physiology. Below we provide specific examples of how breath testing can be used to test a variety of hypotheses in search of new comparative patterns.

Temperature in ectotherms

Many ectotherms experience a wide range of ambient temperatures which has profound effects on their body temperatures and thus their energy demands. While the

thermosensitivity of overall metabolic rate (Q_{10}) is routinely measured and reported across different temperatures [e.g., (Nespolo et al. 2003; Rezende et al. 2010)] the possibility that body temperature affects the oxidative fuel ratios has not been thoroughly examined (Vogt and Appel 1999). Indeed the ability to regulate what nutrients are used for energy and which ones are stored is an important component of the life history of many animals (Boggs 1992). Breath testing on animals as small as crickets can be used to determine whether the relative contributions of different metabolic substrates changes at different temperatures.

In practice, two otherwise identical populations of animals could be raised over the course of their lives on tracers such as ^{13}C-leucine and ^{13}C-palmitic acid to isotopically enrich their body proteins and lipids, respectively. The metabolic rates of these animals will undoubtedly change at different temperatures, and if the δ^{13}C in their breath remains relatively constant across these temperatures (Fig. 6a–c), it can be concluded that temperature does not affect their oxidative fuel mixtures (Fig. 6b, c). However, temperature-induced changes in the δ^{13}C of the breath that do occur indicate that the fuel mixture is temperature dependent. Several possible scenarios are presented in Fig. 6 (Fig. 6d–f). A similar experimental approach could also be used to test whether dehydration (rather than temperature) affects the extent to which ectotherms oxidize different fuels as has been reported in birds (Gerson and Guglielmo 2011a, b).

Temperature in endotherms

Hundreds of endothermic species exhibit heterothermy [reviewed in (Geiser 2004; Hohtola 2012; McKechnie and Mzilikazi 2011; Ruf and Geiser 2014)] which involves reductions in body temperature that may last a couple of hours to several months. While the primary goal of hypothermia is generally believed to conserve energy, the process of arousal can be quite metabolically demanding (Munoz-Garcia et al. 2013; van Breukelen and Martin 2015). Breath testing using populations of animals that have been raised on diets containing ^{13}C-palmitic acid or ^{13}C-leucine to enrich their endogenous lipid or proteins, respectively, could be used to quantify changes in the relative reliance on these fuels while they allow their body temperatures to decrease and then later to upregulate metabolism when they return to euthermy (Fig. 7).

In practice individual animals would be maintained inside a respirometry chamber through which flow rates of CO_2-free air could be regulated (Fig. 1). Metabolic rates would be measured as usual, but a subsample of the excurrent gas could be directed to a ^{13}C-analyzer that is capable of measuring ^{13}C in atmospheric levels (~0.04 %) in real time. If such a ^{13}C-analyzer was unavailable the air flow through the chamber could be paused for long enough to accumulate sufficient levels of CO_2 for analysis using other devices. For example, a euthermic, 150 g golden-mantled ground squirrel at rest inside a 2 L respirometry chamber will generate >2 % CO_2 (concentrations sufficient to be

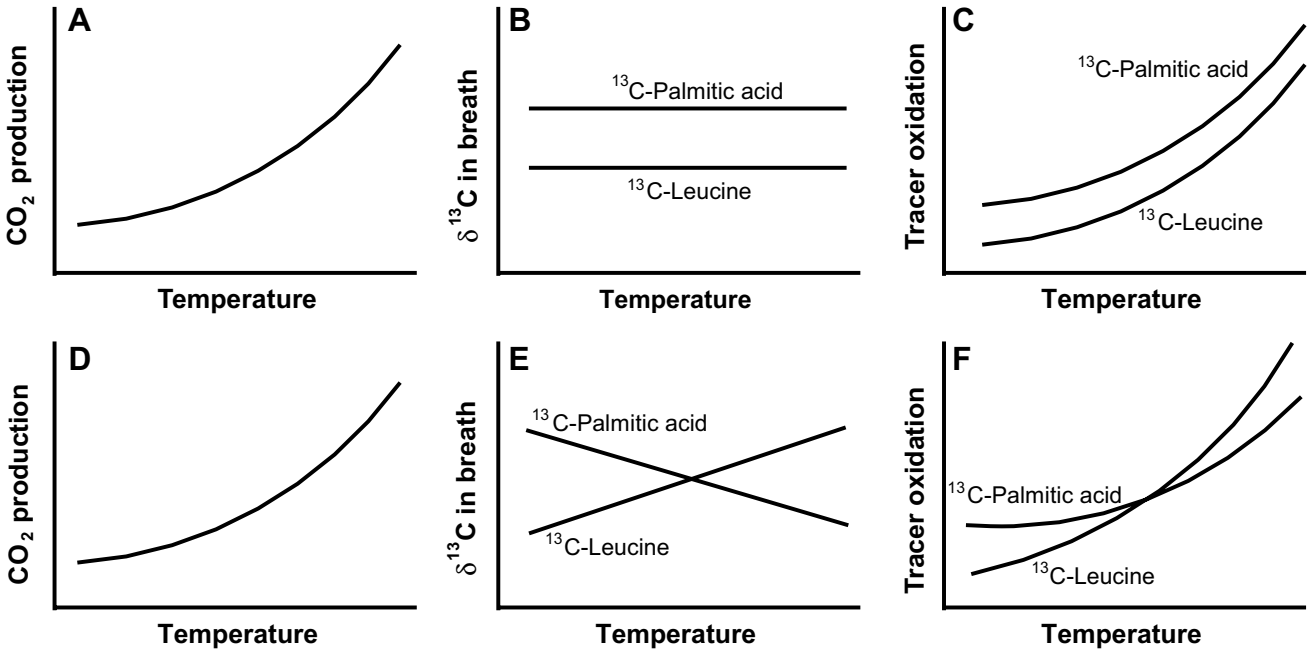

Fig. 6 Hypothetical effects of temperature on metabolic rate and tracer oxidation in ectotherms. **a–c** A scenario where oxidative fuel ratios are temperature insensitive. **d–e** A scenario where oxidative fuel ratios change across temperatures. See text for detailed explanations

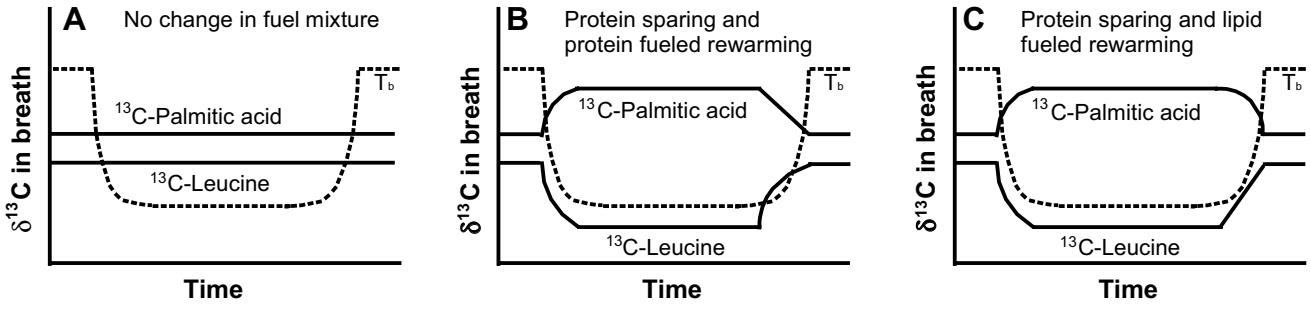

Fig. 7 Hypothetical responses to oxidative fuel mixtures in heterothermic endotherms experiencing changes in body temperature (T_b; *dotted line*). **a** No changes in oxidative fuel mixture. **b** Proteins are spared during cooling and protein is preferentially used to power rewarming. **c** Proteins are spared during cooling and during rewarming. See text for detailed explanations

measured by lower cost nondispersive infrared ^{13}C-analyzers) inside the chamber within 10 min. The same squirrel exhibiting a 10 °C reduction in body temperature would therefore require ~20–30 min to generate similar levels of CO_2 for analysis.

Interpreting the results would be straightforward. If the ratios of protein and lipid oxidation remain constant during the entire heterothermic event, then δ^{13}C values would not change (Fig. 7a). If the animals spared proteins during entry into hypothermia yet used them preferentially to power the arousal phase then the δ^{13}C values would follow the general pattern illustrated in Fig. 7b. If proteins were again spared during the entry phase, but the animals preferentially increased lipid oxidation to fuel the arousal phase then the δ^{13}C profile would show the general pattern illustrated in Fig. 7c. Other outcomes could be possible.

Natural diet shifts

Many animals undergo natural diet shifts during which they transition from consuming one food source to another [e.g., (Hentschel 1998; Phillips and Eldridge 2006; Tallamy and Pesek 1996; Vander Zanden et al. 1998; Willson et al. 2010)]. These shifts may be driven by ontogeny in the case of many holometabolous insects and amphibians that undergo metamorphosis or may be the result of seasonal food availability as seen in many temperate species that are active year-round. While studies report the structural changes in the gut associated with these natural shifts [e.g., (Lignot 2012; Starck 2003)], little is known about how these diet shifts affect how animals use their incoming nutrients to fuel their metabolic demands. Breath testing using different ^{13}C-artifically enriched tracers could be used to characterize the changes in oxidative disposal during these shifts.

One interesting example involves the many migrating birds that exploit seasonal shifts in diet to enhance fat gain at migratory stopover points or prior to departure (Guglielmo 2010). In some instances, these seasonally exploited resources are rich in particular fatty acids (e.g., $n-6$ or $n-3$ polyunsaturated fatty acids) and researchers have correlated increased intake of these lipids with enhanced metabolic performance [e.g., (Guglielmo 2010; Pierce et al. 2005; Price and Guglielmo 2009)]. Yet, the mechanism underlying this improved performance is not fully understood. Maillet and Weber have shown that increased intake of $n-3$ fatty acids in semipalmated sandpipers is associated with possible modifications to membrane fluidity that may enhance fatty acid flux among tissues and increased activity of several key metabolic enzymes (Maillet and Weber 2006, 2007). Yet, while it is commonly understood that migrating birds oxidize fat as their predominant fuel, the identities of the lipids oxidized, and any enhancement of oxidative flux conferred by the use of some lipid classes over others, remain unknown. Indeed, much of the ingested $n-3$ fatty acids is converted to other lipid classes when stored, presumably in order to enhance their energy density or ease of transport from adipose stores to flight muscle (Guglielmo 2010). The ability to track the oxidation of specific ^{13}C-labelled saturated or unsaturated fatty acids (either ingested or injected) using breath testing could thus provide a key complementary approach to unraveling the mechanisms by which 'natural doping' enhances flight performance throughout their marathon journey (Maillet and Weber 2007).

Combined physiological challenges

Researchers have used breath testing to study the responses to a variety of singular physiological and pathophysiological challenges as summarized above, but we are not aware of any studies that have used breath testing to examine the responses to multiple challenges occurring simultaneously. A study of the metabolic rates of varanid lizards showed that exercise-induced VO_{2max} reached a new ceiling when the lizards were simultaneously digesting a large meal

Table 1 A comparison of common technologies used to measure the ^{13}C-content in gas samples

Method of ^{13}C-analysis	$[CO_2]$	Real-time	Cost	Portable
Conventional isotope ratio mass spectrometer	Ambient	No	High	No
Tunable diode laser absorption spectrometer	Ambient	Yes	Medium	No
Cavity ring down spectrometer	Ambient	Yes	Low	Yes
Nondispersive infrared spectrometer	>1 %	No	Low	Yes

Ambient concentrations refer to those similar to atmospheric conditions (e.g., ~400 ppm, 0.04 %). Real time refers to whether the technology can be easily integrated into existing respirometry systems that measure CO_2 production. Cost refers to a combination of equipment procurement and running costs

(Hicks and Bennett 2004) thereby challenging the classic concept of VO_{2max}. How are patterns of fuel oxidation altered under this and similar combined challenges from conditions where the animal is only exercising?

Fasting animals often reduce their metabolic rates to reduce energy expenditure (McCue 2010), but in some cases periods of food limitation may be confounded with thermoregulatory challenges that increase metabolic demand. In the case of endotherms this occurs when ambient temperatures fall outside of the thermal neutral zone [e.g., (Rezende and Bacigalupe 2015; Whiteman et al. 2015)] and for ectotherms, virtually any increase in temperature causes increased metabolic demand [reviewed in (McCue 2004; McNab 2002)]. How do fasting animals adjust the way they mobilize and oxidize their limited resources during these simultaneous challenges? Breath testing can be used to quantify how animals respond to these combined physiological challenges. Other simultaneous stressor pairings could involve food limitation plus exercise, as occurs during some long-distance migration events in birds (Jenni-Eiermann and Jenni 2012; Piersma et al. 2005).

Studying diets of wild animals

Researchers have used the $\delta^{13}C$ in the blood of penguins and the blubber of dolphins to show that groups of individuals within a population exhibit different foraging habits (Cherel and Hobson 2007; Kiszka et al. 2010), but these samples are invasive. Breath testing has recently been employed to gain insights into the food sources used of birds captured at migratory stopovers (Podlesak et al. 2005), vampire bats during returning from their hunt (Voigt 2009), and brown bears undergoing seasonal diet shifts (Whiteman et al. 2012). Breath testing in wild cheetahs demonstrated that it could be used to partition the relative reliance on browsers and grazers as prey items (Voigt et al. 2013). A study of polar bear breath revealed that berries provide a negligible contribution to the energy budget during the summer months (Hobson et al. 2009). Even the breath of insects has been used to show that some grasshoppers actually subsist without eating grass (Engel et al. 2009).

Similar determinations of dietary sourcing could be made on other wild animals using portable ^{13}C-measurement systems (Crosson et al. 2002; Engel et al. 2009; Voigt 2009) and would permit studies of species when blood sampling is not permitted. If portable systems are not feasible a researcher could collect breath samples in a 12 mL Exetainer vial (Fig. 1) and have them analyzed later using tunable diode laser absorption spectroscopy or nondispersive infrared spectroscopy for less than $0.50 USD each (Table 1), or conventional isotope-ratio mass spectroscopy for a couple of dollars per sample (Engel et al. 2009). It is also noteworthy that vials containing 'air' also do not necessarily require any special permitting if transported internationally (J. Ryan Shipley, personal communication).

Conclusion and call for more experiments

While isotopes are subject to physical and chemical rules governing flux through enzymatic reactions the ultimate patterns by which stable isotopes flow through living systems is infinitely complex. Isotope ecologists have made tremendous progress in tracking how carbon moves from the environment through animal tissues, but we maintain that understanding how and when animals oxidize the nutrients that pass through them using breath testing provides critical insight that cannot be revealed from studying tissues alone. The isotope ecology literature over the past decades is replete with pleas for researchers to conduct more controlled studies of stable isotopes in comparative physiology [e.g., (Bearhop et al. 2002; Cherel et al. 2005; Dalerum and Angerbjorn 2005; Gannes et al. 1997; Gorokhova and Haansson 1999; Griffiths 1991; Herrera et al. 2001; Hobson 1999; Martinez del Rio et al. 2009; Phillips and Eldridge 2006; Rubenstein and Hobson 2004; Voigt et al. 2008a; Wise et al. 2006)]. Here we reiterate this plea, but go one step further suggesting that ^{13}C-breath testing needs to be an integral part of this process.

References

Ambrose SH, DeNiro MJ (1986) The isotopic ecology of East African mammals. Oecologia 69:395–406

Amitai O, Holze S, Barkan S, Amichai E, Korine C, Pinshow B, Voigt CC (2010) Fruit bats (Pteropodidae) fuel their metabolism rapidly and directly with exogenous sugars. J Exp Biol 213:2693–2699

Ayliffe LK, Cerling TE, Robinson T, West AG, Sponheimer M, Passey BH, Hammer J, Roeder B, Dearing MD, Ehleringer JR (2004) Turnover of carbon isotopes in tail hair and breath CO_2 of horses fed on an isotopically varied diet. Oecologia 139:11–22

Bammens B, Evenepoel P, Verbake K, Vanrenterghem Y (2004) Impairment of small intestinal protein assimilation in patients with end-stage renal disease: extending the malnutrition-inflammation-atherosclerosis concept. Am J Clin Nutr 80:1536–1543

Barnes C, Sweeting CJ, Jennings S, Barry JT, Polunin NVC (2007) Effect of temperature and ration size on carbon and nitrogen stable isotope trophic fractionation. Funct Ecol 21:356–362

Bauchinger U, McWilliams SR (2009) Carbon turnover in tissues of a passerine bird: allometry, isotopic clocks, and phenotypic flexibility in organ size. Physiol Biochem Zool 82:787–797

Bearhop S, Waldron S, Votier SC, Furness RW (2002) Factors that influence assimilation rates and fractionation of nitrogen and carbon isotopes in avian blood and feathers. Physiol Biochem Zool 75:451–458

Berthold HK, Hatchey DL, Reeds PJ, Thomas OP, Hoeksema S, Klein PD (1991) Uniformly ^{13}C-labeled algal protein used to determine amino acid essentiality in vivo. Proc Natl Acad Sci 88:8091–8095

Bier DM (1997) Stable isotopes in biosciences, their measurement and models for amino acid metabolism. Eur J Pediatr 156:S2–S8

Blair N, Leu A, Munoz E, Olsen J, Kwong E, Marais DD (1985) Carbon isotope fractionation in heterotrophic microbial metabolism. Appl Environ Microbiol 50:996–1001

Bodamer OAF, Halliday D (2001) Uses of stable isotopes in clinical diagnosis and research in the pediatric population. Arch Dis Child 84:444–448

Boggs CL (1992) Resource allocation: exploring connections between foraging and life history. Funct Ecol 6:508–518

Boirie Y, Fauquant J, Rulquin H, Maubois J-L, Beaufrere B (1995) Production of large amounts of [^{13}C] leucine-enriched milk proteins by lactating cows. J Nutr 125:92–98

Boirie Y, Gachon P, Corny S, Fauquant J, Maubois J-L, Beaufrere B (1996) Acute postprandial changes in leucine metabolism as assessed with an intrinsically labeled milk protein. Am J Physiol 271:E1083–E1091

Boirie Y, Dangin M, Gachon P, Vasson M-P, Maubois J-L, Beaufrere B (1997) Slow and fast dietary proteins differently modulate postprandial protein accretion. Proc Natl Acad Sci 94:14930–14935

Bos C, Mahe S, Gaudichon C, Benamouzig R, Guasseres N, Luengo C, Ferriere F, Rautureau J, Tome D (1999) Assessment of net postprandial protein utilization of ^{15}N-labelled milk nitrogen in human subjects. Br J Nutr 81:221–226

Bos C, Metges CC, Gaudichon C, Petzke KJ, Pueyo ME, Morens C, Everwand J, Benamouzig R, Tome D (2003) Postprandial kinetics of dietary amino acids are the main determinant of thier metabolism after soy or milk protein ingestion in humans. J Nutr 133:1308–1315

Braden B (2010) ^{13}C Breath tests for the assessment of exocrine pancreatic function. Pancreas 39:955–959

Brand WA, Coplen TB (2012) Stable isotope deltas: tiny, but robust signatures in nature. Isot Environ Health Stud 48:393–409

Brooks GA (1997) Importance of the "crossover" concept in exercise metabolism. Clin Exp Pharmacol Physiol 24:889–895

Brooks GA, Mercier J (1994) Balance of carbohydrate and lipid utilization during exercise: the "crossover" concept. J Appl Physiol 76:2253–2261

Bujko J, Schreurs VVAM, Nolles JA, Verreijen AM, Koopmanschap E, Verstegen MWA (2007) Application of a [$^{13}CO_2$] breath test to study short-term amino acid catabolism during the postprandial phase of a meal. Br J Nutr 97:891–897

Burelle Y, Lamoureaux M-C, Peronnet F, Massicotte D, Lavoie C (2006) Comparison of exogenous glucose, fructose and galactose oxidation during exercise using ^{13}C-labelling. Br J Nutr 96:56–61

Buyse J, Geypens B, Malheiros RD, Moraes VM, Swennen Q, Decuypere E (2004) Assessment of age-related glucose oxidation rates of broiler chickens by using stable isotopes. Life Sci 75:2245–2255

Carleton SA, Wolf BO, Martinez del Rio C (2004) Keeling plots for hummingbirds: a method to estimate carbon isotope ratios of respired CO_2 in small vertebrates. Oecologica 141:1–6

Carleton SA, Kelly L, Anderson-Sprecher R, Martinez del Rio C (2008) Should we use one-, or multi-compartment models to describe ^{13}C incorporation into animal tissues? Rapid Comm Mass Specrom 22:3008–3014

Castellini MA, Rea LD (1992) The biochemistry of natural fasting at its limits. Experientia 48:575–582

Chatfield J, Citino S, Munson L, Konopka S (2004) Validation of the ^{13}C-urea breath test for use in cheetahs (*Acinonyx jubatus*) with *Heliobacter*. J Zoo Wildl Med 35:137–141

Chen CSW, Welch KC Jr (2014) Hummingbirds can fuel expensive hovering flight completely with either exogenous glucose or fructose. Funct Ecol 28:589–600

Cherel Y, Hobson KA (2007) Geographical variation in carbon stable isotope signatures of marine predators: a tool to investigate their foraging areas in the Southern Ocean. Mar Ecol Prog Ser 329:281–287

Cherel Y, Hobson KA, Hassani S (2005) Isotopic discrimination between food and blood and feathers of captive penguins: implications for dietary studies in the wild. Physiol Biochem Zool 78:106–115

Chown SB, Ahn WS, Antoniewicz MR (2012) Rational design of ^{13}C-labeling experiments for metabolic flux analysis in mammalian cells. BMC Syst Biol 6:43. doi:10.1186/1752-0509-6-43

Coggan AR (1999) Use of stable-isotopes to study carbohydrate and fat metabolism at the whole-body level. Proc Nutr Soc 58:953–961

Cooper JA, Watras AC, Schriver T, Adams AK, Schoeller DA (2010) Influence of dietary fatty acid composition and exercise on changes in fat oxidation from a high-fat diet. J Appl Physiol 109:1011–1018

Coplen TB, Hopple JA, Bohlke JK, Peiser HS, Reider SE, Krouse HR, Rosman KJR, Ding T, Vocke RD, Revessz KM et al. (2002) Compilation of minimum and maximum ratios of selected elements in natually occuring terrestrial materials and reagents. In: USGS (ed)

Craig H (1957) Isotopic standards for carbon and oxygen and correction factors for mass-spectrometric analyses of carbon dioxide. Geochim Cosmochim Acta 12:133–149

Crenn P, Thuillier F, Rakatoambinina B, Rongier M, Darmaun D, Messing B (2000) Duodenal vs. gastric administration of labeled leucine for the study of splanchnic metabolism in humans. J Appl Physiol 89:573–580

Crosson ER, Ricci KN, Richman BA, Chilese FC, Owano TG, Provencal RA, Todd MW, Glasser J, Kachanov AA, Paldus BA et al (2002) Stable isotope ratios using cavity ring-down

spectroscopy: determination of $^{13}C/^{12}C$ for carbon dioxide in human breath. Anal Chem 74:2003–2007
Daenzer M, Petzke JP, Bequette BJ, Metges CC (2001) Whole-body nitrogen and splanchnic amino acid metabolism differ in rats fed mixed diets containing casein or its corresponding amino acid mixture. J Nutr 131:1965–1972
Dalerum F, Angerbjorn A (2005) Resolving temporal variation in vertebrate diets using naturally occuring stable isotopes. Oecologia 144:647–658
De Laeter JR, Bohlke JK, De Bievre P, Hidaka H, Peiser HS, Rosman KJR, Taylor PDP (2003) Atomic weights of the elements: review 2000. Pure Appl Chem 75:683–800
Decombaz J, Arnaud M-J, Milon H, Moesch H, Philippossian G, Thelin AL, Howald H (1983) Energy metabolism of medium-chain triglyerides versus carbohydrates during exercise. Eur J Appl Physiol 52:9–14
Deglaire A, Fromentin C, Fouillet H, Airinei G, Gaudichon C, Boutry C, Benamouzig R, Moughan PJ, Tome D, Bos C (2009) Hydrolysed dietary casein as compared with the intect protein reduces postprandial peripheral, but not whole-body, uptake of nitrogen in humans. Am J Clin Nutr 90:1011–1022
DeNiro MJ, Epstein S (1977) Mechanism of carbon isotope fractionation associated with lipid synthesis. Science 197:261–263
Des Rosiers C, David F, Garneau M, Brunengraber H (1991) Nonhomogeneous labeling of liver mitochondrial acetyl-Co-A. J Biol Chem 266:1574–1578
Doronin YK, Bednik DY, Ivanov AA, Kalistratova EN (2012) Age-dependence of animal metabolism: an insight through the isotope fractionation prism. Sci Domain
Elia M, Fuller N, Murgatroyd P (1988) The potential use of the labelled bicarbonate methods for estimating energy expenditure in man. Proc Nutr Soc 47:247–258
Engel S, Lease HM, McDowell NG, Corbett AH, Wolf BO (2009) The use of tunable diode laser absorption spectroscopy for rapid measurements of delta ^{13}C of animal breath for physiological and ecological studies. Rapid Commun Mass Spectrom 23:1281–1286. doi:10.1002/rcm.4004
Evenepoel P, Hiele M, Luypaerts A, Geypens B, Buyse J, Decuypere E, Rutgeerts P, Ghoos Y (1997) Production of egg proteins, enriched with L-Leucine-$^{13}C_1$, for the study of protein assimilation in humans using the breath test technique. J Nutr 127:327–331
Evenepoel P, Hiele M, Geypens B, Geboes KP, Rutgeerts P, Ghoos Y (2000) ^{13}C-Egg white breath test: a non-invasive test of pancreatic trypsin activity in the small intestine. Gut 46:52–57
Farquhar GD, Ehleringer JR, Hubick KT (1989) Carbon isotope discrimination and photosynthesis. Annu Rev Plant Physiol 40:503–537
Fischer H, Wetzel K (2002) The future of ^{13}C-breath tests. Food Nutr Bull 23:53–56
Focken U, Becker K (1998) Metabolic fractionation of stable carbon isotopes: implications of different proximate compositions for studies of the aquatic food web using $\delta^{13}C$ data. Oecologia 115:337–343
Folch N, Peronnet F, Massicotte D, Duclos M, Lavoie C, Hillaire-Marcel C (2001) Metabolc response to small and large ^{13}C-labelled pasta meals following rest or exercise in man. Brit J Nutr 85:671–680
Fromentin C, Sanders P, Nau F, Anton M, Fromentin G, Tome D, Thibault J-N, Gaudichon C (2012) A pilot study for the intrinsic labeling of egg proteins with ^{15}N and ^{13}C. Rapid Commun Mass Spectrom 26:43–48
Fry B (2006) Stable isotope ecology. Springer, New York
Gannes LZ, O'Brien DM, Martinez del Rio C (1997) Stable isotopes in animal ecology: assumptions, caveats, and a call for more laboratory experiments. Ecology 78:1271–1276
Gautier J-F, Pirnay F, Jandrain B, Lacroix M, Mosora F, Scheen A, Lefebvre PJ (1993) Endogenous substrate oxidation during exercise and variations in breath $^{13}CO_2/^{12}CO_2$. J Appl Physiol 74:133–138
Gay L-J, Schutz Y, DiVetta V, Schneiter P, Tappy L, Jequier E (1994) Measurement of $^{13}CO_2$ in expired air as an index of compliance to a high carbohydrate diet naturally enriched in ^{13}C. Int J Obes 18:591–595
Geboes KP, Bammens B, Luypaerts A, Malheiros RD, Buyse J, Evenepoel P, Rutgeerts P, Verbeke K (2004) Validation of a new test meal for protein digestion breath test in humans. J Nutr 134:806–810
Geiser F (2004) Metabolic rate and body temperature reduction during hibernation and daily torpor. Annu Rev Physiol 66:239–274
Gerson AR, Guglielmo CG (2011a) Flight at low ambient humidity increases protein catabolism in migratory birds. Science 333:1434–1436
Gerson AR, Guglielmo CG (2011b) House sparrows (*Passer domesticus*) increase protein catabolism in response to water restriction. Am J Physiol 300:R925–R930
Ghoos Y, Beaufrere B (1998) ^{13}C Protein breath tests. Gut 43:S23–S24
Goedecke JH, Gibson A, Grobler L, Collins M, Noakes TD, Lambert EV (2000) Determinants of the variability in respiratory exchange ratio at rest and during exercise in trained athletes. Am J Physiol 279:E1325–E1334
Gorokhova E, Haansson S (1999) An experimental study on variations in stable carbon and nitrogen isotope fractionation during growth in *Mysis mixta* and *Neomysis integer*. Can J Fish Aquat Sci 56:2203–2210
Griffiths H (1991) Applications of stable isotope technology in physiological ecology. Funct Ecol 5:254–269
Grusak MA (1997) Intrinsic stable isotope labeling of plants for nutritional investigations in humans. Nutr Biochem 8:164–171
Guglielmo CG (2010) Move that fatty acid: fuel selection and transport in migratory birds and bats. Integr Comp Biol 50:336–345
Guzman RM, McCue MD, Pollock ED, McCue KE (2015) Prolonged fasting causes systematic changes in rats: $^{13}CO_2$ breath testing and small molecule metabolomics. Integr Comp Biol 55:E267
Hall K (2012) Quantitative physiology of human starvation: adaptations of energy expenditure, macronutrient metabolism and body composition. In: McCue MD (ed) Comparative physiology of fasting, starvation, and food limitation. Springer, New York, pp 379–394
Hambly C, Voigt CC (2011) Measuring energy expenditure in birds using bolus injections of ^{13}C-labelled Na-bicarbonate. Comp Biochem Physiol 158A:323–328
Hambly C, Pinshow B, Wiersma P, Verhulst S, Piertney SB, Harper EJ, Speakman JR (2007) Comparison of the cost of short flights in a nectarivorous and a non-nectarivorous bird. J Exp Biol 207:3959–3968
Harlow H (2012) Muscle protein and strength retention by bears during winter fasting and starvation. In: McCue MD (ed) Comparative physiology of fasting, starvation, and food limitation. Springer, New York, pp 277–296
Hatch KA, Pinshow B, Speakman JR (2002) The analysis of $^{13}C/^{12}C$ ratios in exhaled CO_2: its advantages and potential application to field research to infer diet, changes in diet over time, and substrate metabolism in birds. Integr Comp Biol 42:21–33
Hayes JM (2001) Fractionation of carbon and hydrogen isotopes in biosynthetic processes. Rev Mineral Geochem 43:225–277
Hentschel BT (1998) Intraspecific variations in $d^{13}C$ indicate ontogenetic diet changes in deposit-feeding polychaetes. Ecology 79:1357–1370
Herrera LGM, Hobson KA, Manzo AA, Estrada DB, Sanchez-Cordero V, Mendez GC (2001) The role of fruits and insects in the

nutrition of frugivorous bats: evaluating the use of stable isotope models. Biotropica 33:520–528
Hicks JW, Bennett AF (2004) Eat and Run: prioritization of oxygen delivery during elevated metabolic states. Respir Physiol Neurobiol 144:215–224
Hiele M, Ghoos Y, Rutgeerts P, Vantrappen G, de Buyser K (1990) $^{13}CO_2$ breath test to measure the hydrolysis of various starch formulations in healthy subjects. Gut 31:175–178
Hobson KA (1999) Tracing origins and migration of wildlife using stable isotopes: a review. Oecologia 120:314–326
Hobson KA, Yohannes E (2007) Establishing elemental turnover in exercising birds using a wind tunnel: implications for stable isotope tracking of migrants. Can J Zool 85:703–708
Hobson KA, Stirling I, Andriashek DS (2009) Isotopic heterogeneity of breath CO_2 form fasting and berry-eating polar bears: implications of tracing reliance on terrrestrial foods in a changing Arctic. Can J Zool 87
Hoffman JC, Sutton TT (2010) Lipid correction for carbon stable isotope analysis of deep-sea fishes. Deep Sea Res I 57:956–964
Hohtola E (2012) Thermoregulatory adaptations to starvation in birds. In: McCue MD (ed) Comparative physiology of fasting, starvation, and food limitation. Springer, New York, pp 155–170
Hussey NE, Brush J, McCarthy ID, Fisk AT (2010) $\delta^{15}N$ and $\delta^{13}C$ diet-tissue discrimination factors for large sharks under semi-controlled conditions. Comp Biochem Physiol 155A:445–453
Istfan NW, Ling PR, Bistrian BR, Blackburn GL (1988) Systemic exchangeability of enteral leucine: relationship to plasma flux. Am J Physiol 254:R688–R698
Jandrain BJ, Pallikarakis N, Normand S, Pirnay F, Lacroix M, Mosora F, Pachiaudi C, Gautier JF, Scheen AJ, Riou JP et al (1993) Fructose utilization during exercise in men: rapid conversion of ingested fructose to circulating glucose. J Appl Physiol 74:2146–2154
Jenni-Eiermann S, Jenni L (2012) Fasting in birds: general patterns and the special case of endurance flight. In: McCue MD (ed) Comparative physiology of fasting, starvation, and food limitation. Springer, New York, pp 171–192
Jentjens RLPG, Venables MC, Jeukendrup AE (2004a) Oxidation of exogenous glucose, sucrose, and maltose during prolonged cycling exercise. J Appl Physiol 96:1285–1291
Jentjens RLPG, Moseley L, Waring RH, Harding LK, Jeukendrup AE (2004b) Oxidation of combined ingestion of glucose and fructose during exercise. J Appl Physiol 96:1277–1284
Jeukendrup AE, Saris WH, Schrauwen P, Brouns F, Wagenmakers AJ (1995) Metabolic availability of medium-chain triglycerides coingested with carbohydrates during prolonged exercise. J Appl Physiol 79:756–762
Jones PJH, Pencharz PB, Clandinin MT (1985) Whole body oxidation of dietary fatty acids: implications for energy utilization. Am J Clin Nutr 42:769–777
Khalilieh A, McCue MD, Pinshow B (2012) Physiological responses to food deprivation in the house sparrow, a species not adapted to prolonged fasting. Am J Physiol 303:R551–R561
Kiszka J, Oremus M, Richard P, Poole M, Ridoux V (2010) The use of stable isotope analyses from skin biopsy samples to assess trophic relationships of sympatric delphinids off Moorea (French Polynesia). J Exp Mar Biol Ecol 395:48–54
Kleiber M (1975) The fire of life. Krieger, Huntington
Kohno T, Ito A, Hosoi I, Hirayama J, Shibata K (2007) Synthetic ^{13}C-dipeptide breath test for the rapid assessment of pancreatic exocrine insufficiency in rats. Scand J Gastroenterol 42:992–999
Koletzko B, Demmelmair H, Hartl W, Kindermann A, Koletzko S, Sauerwald T, Szitanyi P (1998) The use of stable isotope techniques for nutritional and metabolic research in paediatrics. Early Human Dev 53:S77–S97
Koutsari C, Sidossis LS (2003) Effect of isoenergetic low- and high-carbohydrate diets on substrate kinetics and oxidation in healthy men. Br J Nutr 90:413–418
Kriengsinyos W, Wykes LJ, Ball RO, Pencharz PB (2002) Oral and intravenous tracer protocols of the indicator amino acid oxidation method provide the same estimate of the lysine requirement in healthy men. J Nutr 132:2251–2257
Kuipers H, Keizer HA, Brouns F, Saris WHM (1987) Carbohydrate feeding and glycogen synthesis during exercise in man. Pflugers Arch 410:652–656
Kurle CM, Koch PL, Tershy BR, Croll DA (2014) The effects of sex, tissue type, and dietary components on stable isotope discriminaiton factors ($\Delta^{13}C$ and $\Delta^{15}N$) in mammalian omnivores. Isot Environ Health Stud 50:307–321
Lacroix ML, Mosora F, Pontus M, Lefebvre P, Luyckx A, Lopez-Habib G (1973) Glucose naturally labeled with carbon-13: use for metabolic studies in man. Science 181:445–446
Leslie MA, Coleman RA, Moehn S, Ball RO, Korver DR (2006) Relationship between bicarbonate retention and bone characteristics in broiler chickens. Poult Sci 85:1917–1922
Lighton JRB (2008) Measuring metabolic rates: a manual for scientists. Oxford University Press, New York
Lighton JRB, Halsey LG (2011) Flow-through respirometry applied to chamber systems: pros and cons, hints and tips. Comp Biochem Physiol 158A:265–275
Lignot J-H (2012) Changes in form and function of the gastrointestinal tract during starvation: from pythons to rats. In: McCue MD (ed) Comparative physiology of fasting, starvation, and food limitation. Springer, New York, pp 217–236
Logan JM, Jardine TD, Miller TJ, Bunn SE, Cunjak A, Lutcavage ME (2008) Lipid corrections in carbon and nitrogen stable isotope analyses: comparison of chemical extraction and modelling methods. J Anim Ecol 77:838–846
MacAvoy SE, Arneson LS (2006) Correlation of metabolism with tissue carbon and nitrogen turnover rate in small animals. Oecologia 150:190–201
Macko SA, Estep MF, Hare PE, Hoering TC (1983) Stable nitrogen and carbon isotopic composition of individual amino acids isolated from cultured microorganisms. Carnegie Institution Yearbook 82:404–410
Mahon AD, Timmons BW (2014) Application of stable isotope tracers in the study of exercise metabolism in children: a primer. Pediatr Exerc Sci 26:3–10
Maillet D, Weber J-M (2006) Performance-enhancing role of dietary fatty acids in a long-distance migrant shorebird: the semipalmated sandpiper. J Exp Biol 209:2686–2695
Maillet D, Weber J-M (2007) Relationship between n−3 PUFA content and energy metabolism in the flight muscles of a migrating shorebird: evidence for natural doping. J Exp Biol 210:413–420
Malte CL, Norgaard S, Wang T (2015) Closed system respirometry may underestimate tissue gas exchange and bias the respiratory exchange ratio (RER). Comp Biochem Physiol A **(in press)**
Martinez del Rio C, Carleton SA (2012) How fast and how faithful: the dynamics of isotopic incorporation into animal tissues. J Mammal 93:353–359
Martinez del Rio C, Wolf BO (2005) Mass-balance models for animal isotopic ecology. In: Starck JM, Wang T (eds) Physiological and ecological adaptations to feeding in vertebrates. Science Publishers, Enfield, pp 141–174
Martinez del Rio C, Wolf N, Carleton SA, Gannes LZ (2009) Isotopic ecology ten years after a call for more laboratory experiments. Biol Rev 84:91–111
Massicotte D, Peronnet F, Allah C, Hillaire-Marcel C, Ledoux M, Brisson GR (1986) Metabolic responses to [^{13}C] glucose and [^{13}C] fructose ingestion during exercise. J Appl Physiol 61:1180–1184

Massicotte D, Peronnet F, Brisson GR, Bakkouch K, Hillaire-Marcel C (1989) Oxidation of a glucose polymer during exercise: comparison with glucose and fructose. J Appl Physiol 66:179–183

Massicotte D, Peronnet F, Brisson GR, Hillaire-Marcel C (1992) Oxidation of exogenous medium-chain free fatty acids during prolonged exercise: comparison with glucose. J Appl Physiol 73:1334–1349

Massicotte D, Peronnet F, Pitre C, Adopo E, Brisson G, Hillaire-Marcel C (1993) Exogenous ^{13}C glucose oxidation during exercise: North American vs Western European studies. Eur J Appl Physiol 67:402–407

Matthews DE, Bier DM (1983) Stable isotope methods for nutritional investigation. Annu Rev Nutr 3:309–339

McClelland GB (2004) Fat to the fire: the regulation of lipid oxidation with exercise and environmental stress. Comp Biochem Physiol 139A:443–460

McCloy U, Ryan MA, Pencharz PB, Ross RJ, Cunnane SC (2004) A comparison of the metabolism of eighteen-carbon ^{13}C-unsaturated fatty acids in healthy women. J Lipid Res 45:474–485

McCue MD (2004) General effects of temperature on animal biology. In: Valenzuela N, Lance VA (eds) Temperature dependent sex determination. Smithsonian Books, Washington D.C., pp 71–78

McCue MD (2010) Starvation physiology: reviewing the different strategies animals use to survive a common challenge. Comp Biochem Physiol 156A:1–18

McCue MD (2011) Tracking the oxidative and non-oxidative fates of isotopically labeled nutrients in animals. Bioscience 61:217–230

McCue MD, Cardentey A (2014) Isotope labeled metabolic tracers are not created equal: comparing the oxidative kinetics of free- versus biochemically-integrated dietary nutrients. Integr Comp Biol 54:E136

McCue MD, Pollock ED (2013) Measurements of substrate oxidation using $^{13}CO_2$ breath testing reveals shifts in fuel mix during prolonged fasting. J Comp Physiol 183B:1039–1052

McCue MD, Pinshow B, McWilliams SR (2010a) Tracking exogenous nutrient allocation and routing in postprandial house sparrows with stable isotopes. Physiologist 53:S32

McCue MD, Sivan O, McWilliams SR, Pinshow B (2010b) Tracking the oxidative kinetics of carbohydrates, amino acids, and fatty acids in the house sparrow using exhaled $^{13}CO_2$. J Exp Biol 213:782–789

McCue MD, McWilliams SR, Pinshow B (2011) Ontogeny and nutritional status influence oxidative kinetics of exogenous nutrients and whole-animal bioenergetics in zebra finches, *Taeniopygia guttata*. Physiol Biochem Zool 84:32–42

McCue MD, Arquisola B, Albach E, Pollock ED (2013a) Hens produce artificially enriched ^{13}C egg proteins for metabolic tracer studies. Int J Biol 5:69–84

McCue MD, Amaya JA, Yang AS, Erhardt EB, Wolf BO, Hanson DT (2013b) Targeted ^{13}C enrichment of lipid and protein pools in the body reveals circadian changes in oxidative fuel mixture during prolonged fasting: a case study using Japanese quail. Comp Biochem Physiol 166A:546–554

McCue MD, Voigt CC, Jefimow M, Wojciechowski M (2014) Thermal acclimation and nutritional history affect the oxidation of different classes of exogenous nutrients in Siberian hamsters, *Phodopus sungorus*. J Exp Zool 321:503–514

McCue MD, Passement CA, Guzman RM (2015a) Digesting pythons oxidize the proteins in their meals and save the lipids for later. J Exp Biol 215:2089–2096

McCue MD, Passement CA, Rodriguez M (2015b) The magnitude of the naturally occurring isotopic enrichment of ^{13}C in exhaled CO_2 is directly proportional to exercise intensity in humans. Comp Biochem Physiol 179A:164–171

McCue MD, Guzman RM, Passement CA, Davidowitz G (2015c) How do insects rely on endogenous protein and lipid resources during lethal bouts of starvation? A new application for ^{13}C-breath testing. PlosOne 10:e0140053

McCue MD, Boardman L, Kleynhans E, Terblanche JS (2016) High temperature reduces the energy and duration required for blood meal digestion, but compromises starvation resistance in tsetse flies. Integr Comp Biol **(in press)**

McKechnie AE, Mzilikazi N (2011) Heterothermy in Afrotropical mammals and birds: a review. Integr Comp Biol 51:349–363

McNab BK (2002) The physiological ecology of vertebrates. Cornell University Press, Ithaca

Metges CC, Wolfram G (1991) Medium- and long-chain triglycerides labeled with ^{13}C: a comparison of oxidation after oral or parental administration in humans. J Nutr 121:31–36

Metges CC, El-Khoury AE, Selvaraj AB, Tsay Rh, Atkinson A, Regan MM, Bequette BJ, Young VR (2000) Kinetics of L-[1-^{13}C] leucine when ingested with free amino acids, unlabeled or intrinsically labeled casein. Am J Physiol 278:E1000–E1009

Miller RF, Orr GL, Fritz P, Downer RGH, Morgan AV (1985) Stable carbon isotope ratios in *Periplaneta americana* L., the American cockroach. Can J Zool 63:584–589

Moehn S, Bertolo RFP, Pencharz PB, Ball RO (2004) Pattern of carbon dioxide production and retention is similar in adult pigs when fed hourly, but not when fed a single meal. BMC Physiol 4

Mudambo KSMT, Leese GP, Rennie MJ (1997) Gastric emptying in soldiers during and after field exercise in the heat measured witht he [^{13}C]acetate breath test method. Eur J Appl Physiol 75:109–114

Munoz-Garcia A, Ben-Hamo M, Korine C, Pinshow B, Williams JB (2013) A new thermoregulatory index for heterothermy. Methods Ecol Evol 5:141–145

Murphy EJ (2006) Stable isotope methods for the in vivo measurement of lipogenesis and triglyceride metabolism. J Anim Sci 84:E94–E104

Nespolo RF, Lardies MA, Bozinovic F (2003) Intrapopulational variation in the standard metabolic rate of insects: repeatability, thermal dependence and sensitivity (Q_{10}) of oxygen consumption in a cricket. J Exp Biol 206:4309–4315

Newsome SD, Martinez del Rio C, Bearhop S, Phillips DL (2007) A niche for isotopic ecology. Front Ecol Environ 5:429–436

Newsome SD, Wolf N, Peters J, Fogel ML (2014) Amino acids $\delta^{13}C$ analysis shows flexibility in the routing of dietary protein and lipids to the tissue of an omnivore. Integr Comp Biol 54:890–902

Obeid OA, Powell-Tuck J, Emery PW (2000) The postprandial rates of glycogen and lipid synthesis of lean and obese female Zucker rats. Int J Obes 24:508–513

O'Leary MH (1988) Carbon isotopes in photosynthesis. Bioscience 38:328–336

O'Leary MH, Madhavan S, Paneth P (1992) Physical and chemical basis of carbon isotope fractionation in plants. Plant Cell Environ 15:1099–1104

Oppel S, Federer RN, O'Brien D, Powell AN, Hollmen TE (2010) Effects of lipid extraction on stable isotope ratios in avian egg yolk: is arithmetic correction a reliable alternative? Auk 127:72–78

Papamandjaris AA, White MD, Raeini-Sarjaz M, Jones PJH (2000) Endogenous fat oxidation during medium chain versus long chain triglyceride feeding in healthy women. Int J Obes 24:1158–1166

Park R, Epstein S (1961) Metabolic fractionation of C^{13} & C^{12} in plants. Plant Physiol 36:133–138

Parra MD, Martinez JA (2006) Nutritional aspects of breath testing based on stable isotopes. Nutr Rev 64:338–347

Passey BH, Robinson TF, Ayliffe LK, Cerling TE, Sponheimer M, Dearing MD, Roeder BL, Ehleringer JR (2005) Carbon isotope fractionation between diet, breath CO_2, and bioapetite in different animals. J Archaeol Sci 32:1459–1470

Pate JS (2001) Carbon isotope discrimination and plant water-use efficiency: case scenarios for C_3 plants. In: Unkovich M, Pate J, McNeill A, Gibbs DJ (eds) Stable isotope techniques in the study of biological processes and functioning of ecosystems. Kluwer Academic Publishers, Boston, pp 19–36

Patterson KY, Veillon C (2001) Stable isotopes of minerals as metabolic tracers in human nutrition research. Exp Biol Med 226:271–282

Pauli JN, Ben-David M, Buskirk SW, DePue JE, Smith WP (2009) An isotopic technique to mark mid-sized vertebrates non-invasively. J Zool 278:141–148

Perkins SE, Speakman JR (2001) Measuring natural abundance of ^{13}C in respired CO_2: variability and implications for non-invasive dietary analyses. Funct Ecol 15:791–797

Peronnet F, Adopo E, Massicotte D, Brisson G, Hillaire-Marcel C (1993) Comparison of two methods for computing exogenous substrate oxidation using ^{13}C-labeling. Med Sci Sports Exerc 25:297–302

Phillips DL, Eldridge PM (2006) Estimating the timing of diet shifts using stable isotopes. Oecologia 147:195–203

Phillips SM, Green HJ, Tarnopolsky MA, Heigenhauser GJF, Hill RE, Grant SM (1996) Effect of training duration on substrate turnover and oxidation during exercise. J Appl Physiol 81:2182–2191

Pierce BJ, McWilliams SR, O'Connor TP, Place AR, Guglielmo CG (2005) Effect of dietary fatty acid composition on depot fat and exercise performance in a migrating songbird, the red-eyed vireo. J Exp Biol 208:1277–1285

Piersma T, Perez-Thris J, Mouritsen H, Bauchinger U, Bairlein F (2005) Is there a "migratory syndrome" common to all migrant birds? Ann. N Y Acad Sci 1046:282–293

Podlesak DW, McWilliams SR, Hatch KA (2005) Stable isotopes in breath, blood, feces and feathers can indicate intra-individual changes in the diet of migratory songbirds. Oecologia 142:501–510

Post DM, Layman CA, Arrington DA, Takimoto G, Quattrochi J, Montana CG (2007) Getting to the fat of the matter: models, methods and assumptions for dealing with lipids in stable isotope analyses. Oecologia 152:179–189

Price ER, Guglielmo CG (2009) The effect of muscle phospholipid fatty acid composition on exercise performance: a direct test in the migratory white-throated sparrow (*Zonotrichia albicollis*). Am J Physiol 297:R775–R782

Rating D, Langhans CD (1997) Breath tests: concepts, applications and limitations. Eur J Pediatr 156:S18–S23

Rennie MJ (1999) An introduction to the use of tracers in nutrition and metabolism. Proc Nutr Soc 58:935–944

Rezende EL, Bacigalupe LD (2015) Thermoregulation in endotherms: physiological principles ad ecological consequences. J Comp Biochem 185(7):709–727

Rezende EL, Tejedo M, Santos M (2010) Estimating the adaptive potential of critical thermal limits: methodological problems and evolutionary implications. Funct Ecol 25:111–121

Romijn JA, Coyle EF, Hibbert J, Wolfe RR (1992) Comparison of indirect calorimetry and a new breath $^{13}C/^{12}C$ ratio method during strenuous exercise. Am J Physiol 26:E64–E71

Rubenstein DR, Hobson KA (2004) From birds to butterflies: animal movement patterns and stable isotopes. Trends Ecol Evol 19:256–263

Ruess L, Tiunov A, Haubert D, Richnow HH, Haggblom MM, Scheu S (2005) Carbon stable isotope fractionation and trophic transfer of fatty acids in fungal based soil food chains. Soil Biol Biochem 37:945–953

Ruf T, Geiser F (2014) Daily torpor and hibernation in birds and mammals. Biol Rev 90(3):891–926

Saris WHM, Goodpaster BH, Jeukendrup AE, Brouns F, Halliday D, Wagenmakers AJM (1993) Exogenous carbohydrate oxidation from different carbohydrate sources during exercise. J Appl Physiol 75:2168–2172

Satabin P, Portero P, Defer G, Bricout J, Guezennec CY (1987) Metabolic and hormonal responses to lipid and carbohydrate diets during exercise in man. Med Sci Sports Exerc 19:218–223

Schoeller DA, Klein PD, Watkins JB, Heim T, MacLean WC (1980) ^{13}C abundances of nutrients and the effect of variations in ^{13}C isotopic abundances of test meals formulated for $^{13}CO_2$ breath tests. Am J Clin Nutr 33:2375–2385

Secor SM, Diamond J (1998) A vertebrate model of extreme physiological regulation. Nature 395:659–662

Sidossis LS, Coggan AR, Gastaldelli A, Wolfe RR (1995) A new correction factor for use in tracer estimations of plasma fatty acid oxidation. Am J Physiol 269:E649–E656

Slater C, Preston T, Weaver LT (2001) Stable isotopes and the international system of units. Rapid Commun Mass Spectrom 15:1270–1273

Smith J-EW, Zachwieja JJ, Peronnet F, Passe DH, Massicotte D, Lavoie C, Pascoe DD (2010) Fuel selection and cycling endurance performance with ingestion of [^{13}C] glucose: evidence for a carbohydrate dose response. J Appl Physiol 108:1520–1529

Sonko BJ, Fennessey PV, Donnelly JE, Bessesen D, Sharp TA, Jacobsen DJ, Jones RH, Hill JO (2005) Ingested fat oxidation contributes 8% of 24-h total energy expenditure in moderately obese subjects. J Nutr 135:2159–2165

Speakman JR, Thomson SC (1997) Validation of the labeled bicarbonate technique for measurement of short-term energy expenditure in the mouse. Z Ernahrungswiss 36:273–277

Sponheimer M, Robinson TF, Cerling TE, Tegland L, Roeder BL, Ayliffe L, Dearing MD, Ehleringer JR (2006) Turnover of stable carbon isotopes in the muscle, liver, and breath CO_2 of alpacas (*Lama pacos*). Rapid Commun Mass Spectrom 20:1395–1399

Starck JM (2003) Shaping up: how vertebrates adjust their digestive system to changing environmental conditions. Anim Biol 53:245–257

Starck JM, Moser P, Werner RA, Linke P (2004) Pythons metabolize prey to fuel the response to feeding. Proc R Soc Lond 271B:903–908

Stoll B, Henry J, Reeds PJ, Yu H, Jahoor F, Burrin DG (1998) Catabolism dominates the first-pass intestinal metabolism of dietary essential amino acids in milk-protein fed piglets. J Nutr 128:606–614

Swennen Q, Laroye C, Janssens G, Verbeke K, Decuypere E, Buyse J (2007) Rate of metabolic decarboxylation of leucine as assessed by a L[1-$^{13}C_1$] leucine breath testing combined with indirect calorimetry of broiler chickens fed isocaloric diets with different protein:fat ratio. J Anim Physiol Anim Nutr 91:347–354

Tallamy DW, Pesek JD (1996) Carbon isotope signatures of elytra reflect larval diet in luperine rootworms (Coleoptera: Chrysomelidae). Physiol Chem Ecol 25:1167–1172

Tanis AA, van den Berg JWO, Kroneman BR, Wattimena JLD, Rietveld T, Nieland BHB, Swart GR (1998) Human liver glycogen metabolism assessed with a ^{13}C-enriched diet and a $^{13}CO_2$ breath test. Euro J Clin Invest 28:466–474

Tanis AA, Rietveld T, Van den Berg JWO, Wattimena JLD, Swart GR (2000) Influence of the ^{13}C-enrichment of the habitual diet on a $^{13}CO_2$ breath test used as an index of liver glycogen oxidation: a validation study in Western Europe and Africa. Nutrition 16:6–10

Tanis AA, Rietveld T, Wattimena JLD, van den Berg JWO, Swart GR (2003) The $^{13}CO_2$ breath test for liver glycogen oxidation after

3-day labeling of the liver with a naturally ^{13}C-enriched diet. Nutrition 19:432–437

Thompson DR, Bury SJ, Hobson KA, Wassenaar LI, Shannon JP (2005) Stable isotopes in ecological studies. Oecologia 144:517–519

Trimmer JK, Casazza GA, Horning MA, Brooks JR (2001) Recovery of $^{13}CO_2$ during rest and exercise after [1-^{13}C] acetate, [2-^{13}C], and $NaH^{13}CO_3$ infusions. Am J Physiol 281:E683–E692

van Breukelen F, Martin SL (2015) The hibernation continuum: physiological and molecule aspects of metabolic plasticity in mammals. Physiology 30:273–281

Van Der Merwe NJ (1982) Carbon isotopes, photosynthesis, and archaeology. Am Sci 70:209–215

Van Loon LJC, Jeukendrup AE, Saris WHM, Wagenmakers JM (1999) Effect of training status on fuel selection during submaximal exercise with glucose ingestion. J Appl Physiol 87:1413–1420

Vander Zanden MJ, Hulshof M, Ridgway MS, Rasmussen JB (1998) Application of stable isotope techniques to trophic studies of age-0 smallmouth bass. Trans Am Fish Soc 127:729–739

Venables MC, Achten J, Jeukendrup AE (2005) Determinants of fat oxidation during exercise in healthy men and women: a cross-sectional study. J Appl Physiol 98:160–167

Vogt JT, Appel AG (1999) Standard metabolic rate of the fire ant, *Solenopsis invicta* Buren: effects of temperature, mass, and caste. J Insect Physiol 45:655–666

Voigt CC (2009) Studying animal diets in situ using portable stable isotope analyzers. Biotropica 41:271–274

Voigt CC, Speakman JR (2007) Nectar-feeding bats fuel their high metabolism directly with exogenous carbohydrates. Funct Ecol 21:913–921

Voigt CC, Baier L, Speakman JR, Siemers BM (2008a) Stable carbon isotopes in exhaled breath as tracers for dietary information in birds and mammals. J Exp Biol 211:2233–2238

Voigt CC, Grasse P, Rex K, Hetz SK, Speakman JR (2008b) Bat breath reveals metabolic substrate use in free-ranging vampires. J Comp Physiol 178B:9–16

Voigt CC, Sorgel K, Dechmann DKN (2010) Refuelling while flying: foraging bats combust food rapidly and directly to power flight. Ecology 91:2908–2917

Voigt CC, Sorgel K, Suba J, Keiss O, Petersons G (2012) The insectivorous bat *Pipistrellus nathusii* uses a mixed-fuel strategy to power autumn migration. Proc Roy Soc B 279:3772–3778

Voigt CC, Melzheimer J, Thalwitzer S, Wachter B (2013) A breath test to assign carnivore diets to browsers or grazers. Wildl Biol 19:311–316

Waas S, Werner RA, Starck JM (2010) Fuel switching and energy partitioning during the postprandial metabolic response in the ball python. J Exp Biol 213:1266–1271

Wagenmakers AJM, Rehrer NJ, Brouns F, Saris WHM, Halliday D (1993) Breath $^{13}CO_2$ background enrichment during exercise: diet-related differences between Europe and America. J Appl Physiol 74:2353–2357

Waki H, Watanable K, Ishii H, Tanaka Y, Shumiya S, Ando S (2004) Whole-body β-oxidation of docosahexaenoic acid is decreased under n-3 polyunsaturated fatty acid insufficiency as assessed by a $^{13}CO_2$ breath test using [U-^{13}C] fatty acids. J Oleo Sci 53:135–142

Walsberg GE, Wolf BO (1995) Variation in the respiratory quotient of birds and implications for indirect calorimetry using measurements of carbon dioxide production. J Exp Biol 198:213–219

Warne RW, Gilman CA, Wolf BO (2010) Tissue-carbon incorporation rates in lizards: implications for ecological studies using stable isotoeps in terrestrial ectotherms. Physiol Biochem Zool 83:608–617

Welch KC Jr, Chen CCW (2014) Sugar flux through the flight muscle of hovering vertebrate nectarivores: a review. J Comp Physiol 184B:945–959

Welch KC Jr, Suarez RK (2007) Oxidation rate and turnover of ingested sugar in hovering Anna's (*Calypte anna*) and rufous (*Selasphorus rufus*) hummingbirds. J Exp Biol 210:2154–2162

Welch KC Jr, Bakken BH, Martinez del Rio C, Suarez RK (2006) Hummingbirds fuel hovering flight with newly ingested sugar. Physiol Biochem Zool 79:1082–1087

Welch KC Jr, Gerardo-Herrera L, Suarez RK (2008) Dietary sugar as a direct fuel for flight in the nectarivorous bat *Glossophaga sorincia*. J Exp Biol 211:310–316

Welch KC, Perronet F, Voigt CC, Hatch K, McCue MD (2015) Combining respirometry with stable isotopes to investigate fuel use in animals. Ann N Y Acad Sci. doi:10.1111/nyas.12737 **(in press)**

Whigham LD, Butz DE, Johnson LK, Schoeller DA, Abbott DH, Porter WP, Cook ME (2014) Breath carbon stable isotope ratios identify changes in energy balance and substrate utilization in humans. Int J Obes 38:1248–1250

Whiteman JP, Greller KA, Harlow HJ, Felicetti LA, Rode KD, Ben-David M (2012) Carbon isotopes in exhaled breath track metabolic substrates in brown bears (*Ursus arctos*). J Mammal 93:413–421

Whiteman JP, Harlow HJ, Durner GM, Anderson-Sprecher R, Albeke SE, Regehr EV, Amstrup SC, Ben-David M (2015) Summer declines in activity and body temperature offer polar bears limited energy savings. Nature 349:295–298

Willson JD, Winne CT, Pilgrim MA, Romanek CS, Gobbons JW (2010) Seasonal variation in terrestrial resources subsidies influences trophic niche width and overlap in two aquatic snake species: a stable isotope approach. Oikos 119:1161–1171

Wise DH, Moldenhauer DM, Halaj J (2006) Using stable isotopes to reveal shifts in prey consumption by generalist predators. Ecol Appl 16:865–876

Wolf BO, Hatch KA (2011) Aloe nectar, birds and stable isotopes: opportunities for quantifying trophic interactions. Ibis 153:1–3

Wolfe RR, Chinkes DL (2005) Isotope tracers in metabolic research: principles and practice of kinetic analysis. Wiley, Hoboken

Wolfe RR, Shaw JHF, Nadel ER, Wolfe MH (1984) Effect of substrate intake and physiological state on background $^{13}CO_2$ enrichment. J Appl Physiol 56:230–234