Comparative Biochemistry and Physiology, Part A 209 (2017) 10–15

Contents lists available at ScienceDirect

Comparative Biochemistry and Physiology, Part A

journal homepage: www.elsevier.com/locate/cbpa

Stable isotope analysis of CO_2 in breath indicates metabolic fuel shifts in torpid arctic ground squirrels

Trixie N. Lee[a,*], Melanie M. Richter[b,1], Cory T. Williams[a,2], Øivind Tøien[a], Brian M. Barnes[a], Diane M. O'Brien[a], C. Loren Buck[b,2]

[a] Institute of Arctic Biology, 311 Irving I, University of Alaska Fairbanks, Fairbanks, AK 99775, USA
[b] Department of Biological Sciences, University of Alaska Anchorage, 3101 Science Circle, Anchorage, AK 99508, USA

ARTICLE INFO

Keywords:
Arctic ground squirrel
Breath carbon isotopes
Hibernation
Metabolic fuel
Stable isotopes

ABSTRACT

Stable carbon isotope ratios ($\delta^{13}C$) in breath show promise as an indicator of immediate metabolic fuel utilization in animals because tissue lipids have a lower $\delta^{13}C$ value than carbohydrates and proteins. Metabolic fuel consumption is often estimated using the respiratory exchange ratio (RER), which has lipid and carbohydrate boundaries, but does not differentiate between protein and mixed fuel catabolism at intermediate values. Because lipids have relatively low $\delta^{13}C$ values, measurements of stable carbon isotopes in breath may help distinguish between catabolism of protein and mixed fuel that includes lipid. We measured breath $\delta^{13}C$ and RER concurrently in arctic ground squirrels (*Urocitellus parryii*) during steady-state torpor at ambient temperatures from −2 to −26 °C. As predicted, we found a correlation between RER and breath $\delta^{13}C$ values; however, the range of RER in this study did not reach intermediate levels to allow further resolution of metabolic substrate use with the addition of breath $\delta^{13}C$ measurements. These data suggest that breath $\delta^{13}C$ values are 1.1‰ lower than lipid tissue during pure lipid metabolism. From RER, we determined that arctic ground squirrels rely on nonlipid fuel sources for a significant portion of energy during torpor (up to 37%). The shift toward nonlipid fuel sources may be influenced by adiposity of the animals in addition to thermal challenge.

1. Introduction

Animals use three primary metabolic fuels to meet energy demands: protein, carbohydrate, and lipid. Protein is typically reserved for structural and functional roles and does not contribute significantly to energy metabolism during periods of energy balance (Robbins, 2001). Carbohydrates are often directly metabolized for immediate energy and are not stored in large quantities in mammals (Vock et al., 1996). Lipid is the most energy-dense fuel, providing 8 to 10-times more energy on a wet mass basis than carbohydrate or protein (McWilliams et al., 2004). Thus, lipid, which is stored as white adipose tissue, is used over relatively long time frames after carbohydrate stores are depleted. Several factors influence which fuels are selected for metabolism, including exercise intensity, training status, and diet (reviewed in Holloszy et al., 1998). Animals enduring fasting or starvation exhibit a well-defined pattern of metabolic fuel selection, with timing of shifts between fuels based on the amount of lipid available and catabolism of protein as a last resort (Robbins, 2001).

Mammalian hibernation is a strategy used by a variety of species to conserve energy in anticipation of and during periods of insufficient food resources. During hibernation, animals often do not eat for months and rely entirely on endogenous stores of metabolic fuel. Some animals can conserve as much as 90% of the energy they would otherwise use by spending most of the hibernation season in torpor, the low-metabolism, energy-saving phase of hibernation (Karpovich et al., 2009; Wang and Wolowyk, 1988). Most hibernators support their metabolic demand from large lipid stores which are accumulated during the pre-hibernation fattening period (Dark, 2005), but this appears insufficient for animals hibernating in extreme environmental conditions, such as the Arctic (Buck and Barnes, 2000).

Arctic ground squirrels (*Urocitellus parryii*) are the most northern small hibernator in North America and experience hibernacula temperatures averaging −8.9 °C and as low as −23 °C over the 6–8 month hibernation season (Buck and Barnes, 1999b). Arctic ground squirrels defend a minimum body temperature as low as −2.9 °C for weeks at a time during torpor (Barnes, 1989), which requires them to be con-

Abbreviations: $\delta^{13}C$, carbon isotope ratio; RQ, respiratory quotient; RER, respiratory exchange ratio; T_a, ambient temperature

* Corresponding author at: Biology Department, Box 12251, Harding University, Searcy, AR 72149, USA.
E-mail address: tnlee@harding.edu (T.N. Lee).
[1] Present Address: Biology Department, 1906 College Heights Blvd., #11080, Western Kentucky University, Bowling Green, KY 42101, USA.
[2] Present Address: Department of Biological Sciences and Center for Bioengineering Innovation; 617 S. Beaver St., Northern Arizona University, Flagstaff, AZ 86011-5640, USA.

http://dx.doi.org/10.1016/j.cbpa.2017.04.004
Received 16 January 2017; Received in revised form 4 April 2017; Accepted 5 April 2017
Available online 07 April 2017
1095-6433/

tinuously thermogenic due to the still lower burrow temperatures (Buck et al., 2008). During the short active season, arctic ground squirrels increase lipid (Buck and Barnes, 1999a) and lean mass (Boonstra et al., 2011; Sheriff et al., 2013), and significant proportions of both tissues are used over winter, as indicated by body composition estimates before and after hibernation (Buck and Barnes, 1999b). Use of lean mass during hibernation is also indicated by a shift toward increased reliance on mixed fuel metabolism during torpor at decreasing ambient temperatures (T_a), as indicated by respirometry (Buck and Barnes, 2000). Carbohydrate (glucose) is needed to facilitate lipid oxidation associated with brown adipose tissue thermogenesis (Cannon and Nedergaard, 1979; Vallerand et al., 1990), to support anaplerosis (Owen et al., 2002), and to fuel certain organs such as the kidneys (Berg et al., 2002). The primary precursor to glucose in most hibernators is glycerol, a byproduct of triglyceride catabolism (Galster and Morrison, 1975; Staples and Hochachka, 1998). However, it has been suggested that the increasing demand for carbohydrates by thermogenesis surpasses the supply of glucose formed from glycerol and requires the additional breakdown of protein for gluconeogenesis (Buck and Barnes, 2000; Galster and Morrison, 1975; Krilowicz, 1985). This hypothesis is supported by dramatic upregulation during hibernation of the gene *PCK1*, which codes for a crucial enzyme in gluconeogenesis from pyruvate, lactate, or amino acid precursors (Williams et al., 2011). Whether amino acids are catabolized directly for fuel use during torpor has not yet been determined.

Respirometry is the traditional method used to differentiate metabolic fuel use, but it cannot differentiate between protein and mixed fuel catabolism. The respiratory quotient (RQ), the ratio of carbon dioxide (CO_2) produced to oxygen (O_2) consumed (Kleiber, 1961), is approximately 0.7 during lipid oxidation while carbohydrate oxidation results in an RQ of 1.0. Oxidation of proteins yields an intermediate RQ of 0.83 (Kleiber, 1961), similar to that expected from mixed lipid and carbohydrate metabolism. Respiratory exchange ratios (RER), collected from whole-animal respirometry, are used in this study to approximate RQ values, and we will discuss RQ and RER as a singular concept for the purposes of this study. Alternative measures of fuel use in addition to RQ may help to better distinguish the proportions of metabolic contribution among carbohydrate, protein, and lipid.

Stable carbon isotope measurements are becoming more common in studies of substrate use, as respired CO_2 is a direct product of the animal's metabolism (Hatch et al., 2002; Voigt et al., 2008a; reviewed in: McCue and Welch, 2016; Welch et al., 2016). Lipid has a lower carbon isotope ratio ($\delta^{13}C$) than other metabolites (DeNiro and Epstein, 1977; reviewed in McCue and Welch, 2016). Animals that are fasting shift toward lower $\delta^{13}C$ values in respired CO_2 (Perkins and Speakman, 2001; Schoeller et al., 1984; Voigt et al., 2008a, 2008b), which is consistent with an increase in the proportion of lipid utilization during food deprivation (McCue and Pollock, 2013; Robbins, 2001). Investigations of metabolism in plants have shown a strong correlation between RQ and $\delta^{13}C$ values in respired CO_2 (Pataki, 2005; Tcherkez et al., 2003), but few studies in animals have combined measurements of RER and breath $\delta^{13}C$ values from naturally distinct, endogenous substrates (Gautier et al., 1996; Schoeller et al., 1984).

Metabolic processes have the potential to preferentially use one form of isotope over others. This discrimination can lead to differences in the $\delta^{13}C$ values between fuel source and exhaled CO_2. Several experimental studies have found differences between $\delta^{13}C$ values in breath and diet (discrimination factors; surveyed in Table 2 in Voigt et al., 2008a), but the number of metabolic steps that exogenous and endogenous substrates go through before utilization are different and thus differ in their potential for discrimination. In addition, discrimination in the breakdown of endogenous protein and lipid stores likely varies due to the differences in metabolic pathways, but most metabolic processes have not been thoroughly investigated for evidence of discrimination.

Our first objective was to determine whether RER and $\delta^{13}C$ values covary in an animal system utilizing endogenous substrates with naturally distinct $\delta^{13}C$ signatures. To address this objective, we concurrently measured RER and breath $\delta^{13}C$ values in fasting and hibernating arctic ground squirrels, using changes in T_a to induce shifts in fuel use. Previous work on torpid arctic ground squirrels showed a robust, linear increase in RER as T_a decreased below 0 °C (Buck and Barnes, 2000) and a clear difference in $\delta^{13}C$ values between tissue lipids and lean mass (Lee et al., 2012). Our second objective was to determine whether using two 2-endpoint measurements, RER and breath $\delta^{13}C$ values, could help resolve fuel use in torpid arctic ground squirrels. Specifically, if RER values are intermediate and $\delta^{13}C$ values are intermediate, we would conclude that squirrels are using a mix of lipid and other fuels. However, if RER values are intermediate and $\delta^{13}C$ values are high, we would conclude that the animals are using a fuel based on proteins and/or carbohydrates but utilizing little, if any, lipid. Our final objective was to determine whether there was evidence of discrimination between endogenous fuels and breath $\delta^{13}C$ values.

2. Materials and methods

2.1. Animals

Arctic ground squirrels (*Urocitellus parryii*) were captured near Toolik Field Station (68° 38′ N, 149° 36′ W) in the Alaskan Arctic in fall 2008 and summer 2009 and maintained on Mazuri Rodent Chow in captivity at the University of Alaska Anchorage. Each animal had a temperature-sensitive radiotransmitter surgically implanted in its abdomen (~7 g; Data Sciences International, St. Paul, MN, USA, see Richter et al., 2015 for methods). Ground squirrels were initially held at room temperature on an 18L:6D light cycle in 48 × 32 × 32 cm hanging metal cages and were provided ample cotton material from which they constructed nests. They were then moved into environmental chambers at + 2 °C on a 9L:15D light cycle. When an animal began hibernating (body temperature ≤ 30 °C; Buck et al., 2008), it was transferred to a plastic metabolic chamber with a wire lid and placed on a receiver linked to an automated data collection system (Data Sciences International, St Paul, MN, USA) that recorded core abdominal temperature every 10 min.

2.2. Treatment groups

Once a sufficient number of animals began hibernating, they were divided into two treatment groups that followed different schedules of decreasing T_a. The temperature within Chamber 1 (n = 8 animals, 5 males and 3 females) was decreased from + 2 to − 20 °C (this chamber's minimum temperature) in 2 °C increments. The temperature within Chamber 2 (*n* = 9 animals, 6 males and 3 females) was decreased from + 2 °C to 0 °C, then to − 10 °C, and then to − 20 °C, at which point the temperature was lowered in 2 °C increments until − 26 °C, the minimum T_a at which arctic ground squirrels can sustain steady-state torpor (see Richter et al., 2015). This regime allowed us to measure squirrels that still had adequate lipid reserves at T_a < − 20 °C. At each T_a, RER for each animal in the chamber was recorded for 6 h during steady-state torpor (four animals simultaneously; defined in Respirometry section), and excurrent air containing breath from the respirometry chamber was sampled simultaneously. Once all animals in the environmental chamber had been sampled (typically over a period of several days), all were handled and weighed. This handling induced the squirrels to arouse and begin increasing body temperature, ultimately leading to euthermic body temperature. Once squirrels had been handled and movement and/or rapid breathing was observed, they were returned to the environmental chamber set at the next T_a. Here they completed warming and the period of euthermy of an arousal bout (during which they adjusted to the new T_a), then returned to torpor. All animal use procedures were approved by the University of Alaska Anchorage Institutional Animal Care and Use Committee (pro-

tocol 150918) and follow ARRIVE guidelines.

2.3. Respirometry

Open-system respirometry was used to measure metabolic rate (reported in Richter et al., 2015) and RER of four animals simultaneously during steady-state torpor according to methods by Tøien (2013). Data collection began at least 7 h after body temperature decreased below 0 °C. For all experimental animals, body temperature fluctuated < 0.2 °C over the 6 h of data collection. Metabolic chambers (43 × 27 × 19 cm, 22,059 cm^3) were sealed with weatherstripping on weighted lids (placed on the metabolic chambers of the torpid animals to be measured). Incurrent and excurrent ports were in the chamber lid; however, excurrent air was sampled from tubing that extended from the excurrent port to near the bottom of the chamber to ensure air mixing. Ambient air was drawn through the chamber at 200 ml/min or 2500 ml/min, measured with a flow control unit (Flowbar 8, Sable Systems, Las Vegas, NV, USA) or a Brooks 5851E mass flow controller (Coastal Instruments Inc. Burgaw, NC, USA), respectively, and rate was changed by computer-controlled baselining units (Sable Systems, Las Vegas, NV, USA). Flow rate depended on the metabolic rate of the animal: when O_2 extraction by the animal decreased below 0.08% the system automatically switched to low flow rate, while an increase in O_2 extraction above 1.3% caused a switch to the high flow rate to sustain higher metabolic rates. Flow rate switched to high flow for most animals during arousal episodes and during torpor at − 16 °C, but the change in flow rate did not affect RER measurements (high flow: 0.748 ± 0.009 SD, low flow: 0.743 ± 0.006 SD; $n = 6$, paired t-test: $t = -1.01$, $p = 0.179$).

Excurrent air was analyzed for CO_2 concentrations by two 1-channel CA-10A CO_2 analyzers and O_2 concentrations by a 2-channel Oxzilla II O_2 analyzer (Sable Systems, Las Vegas, NV, USA). The sample stream to each set of analyzer channels was switched between two animals every 5 min with a modified multiplexer (Mux-3, Sable Systems, Las Vegas, NV, USA), resulting in measurements on four animals per trial. Each metabolic trial was preceded by manual span and zero gas calibrations of the instruments. The CO_2 analyzers were calibrated with a span gas of 0.5% CO_2 and a zero gas supplied by passing air through a soda lime scrubber to remove CO_2. The O_2 analyzer was calibrated with a span gas of standard 20.94% air. Span and zero gas values were also automatically recorded every 3 h (for 4 min) during a trial to allow span correction, and ambient CO_2 and O_2 concentrations were recorded every hour (for 4 min). O_2 consumption and CO_2 production were calculated according to the Haldane transformation (Haldane, 1912; Luft et al., 1973; Wagner et al., 1973) with the data acquisition software LabGraph (Tøien, 2013). After sufficient time had elapsed for several complete air changes within the metabolic chambers (6 h at low flow, 2 h at high flow), RER was calculated from the final 6 h of a trial as the average CO_2 produced/O_2 consumed; this sampling scheme averaged RER over a long period to ensure that RER measurements did not reflect short-term storage or release of CO_2 (Bickler, 1984; Snapp and Heller, 1981). Integrity of the respirometry system was assessed by periodic 100% ethanol burns of known RQ (0.6667, Tøien, 2013).

2.4. Breath sample collection and analysis

Excurrent air from the metabolic chambers and reference ambient air pulled directly from the environmental chamber were subsampled upstream of the analyzers via 4-way stopcocks (product #30600-03, Cole Parmer, Vernon Hills, IL, USA) into evacuated glass Exetainers (12 ml, LabCo Limited, Buckinghamshire, UK). CO_2 concentrations of the samples at the time of sampling were measured by the CO_2 analyzers and corrected for any drift in span or offset by LabGraph.

Excurrent air samples were analyzed within 7 weeks by the Alaska Stable Isotope Facility at the University of Alaska Fairbanks for $\delta^{13}C$ values via continuous flow isotope ratio mass spectrometry using a ThermoElectron GasBench II interfaced to a Finnigan Delta^{Plus} XP isotope ratio mass spectrometer (Thermo Fisher Scientific, Bremen, Germany). All isotope values are expressed in delta notation as $\delta^{13}C = ((Rsample - Rstandard) / Rstandard) * 1000‰$, where R = the ratio of heavy to light isotope, and the standard is Vienna PeeDee Belemnite. We used a basic mixing model equation to account for CO_2 present in ambient air:

$$\delta^{13}C_{breath} = (\delta^{13}C_{sample} - (f_{ambient} * \delta^{13}C_{ambient}))/(1 - f_{ambient}) \quad (1)$$

where $f_{ambient}$ is the fraction of ambient air included in the sample. We calculated $f_{ambient}$ as the concentration of CO_2 in ambient air divided by the concentration of CO_2 within the metabolic chamber. A large number of samples from animals held at $T_a > -2$ °C had too little CO_2 contributed by squirrel metabolism for confident determination of squirrel breath $\delta^{13}C$ values (i.e., $f_{ambient} > 0.525$, Welch et al., 2006); therefore, RER and $\delta^{13}C$ values from samples at T_a 0 °C and + 2 °C were not included in the analysis.

To estimate discrimination between lipid fuel and breath CO_2, we compared breath $\delta^{13}C$ values with expected endogenous lipid $\delta^{13}C$ values when RER indicated pure lipid metabolism (RER = 0.707, Kleiber, 1961). Expected lipid $\delta^{13}C$ values for arctic ground squirrels are from five arctic ground squirrels maintained on the same diet as adults in the present study (− 24.8‰ based on the average of two white adipose tissues per animal, n = 10 samples; see details in Lee et al., 2012). Expected muscle $\delta^{13}C$ values, used to approximate nonlipid tissue, were based on lipid-extracted skeletal muscle samples (four muscles from each of five animals, n = 20 samples, $\delta^{13}C = -21.6‰$, Lee et al., 2012).

2.5. Statistical analysis

We determined the relationship between RER and breath $\delta^{13}C$ values with a linear mixed model with $\delta^{13}C$ values as the dependent variable, RER as the independent variable, and individual as a random effect. In each chamber, we compared mean RER and breath $\delta^{13}C$ values at each T_a using a mixed-model ANOVA with individual as a random effect. Four outliers, which were identified as data points > 3 SD from the mean, were eliminated from analysis. To determine the proportions of different endogenous fuels being used from RER, we calculated the proportion of carbohydrate as fuel with an equation from Kleiber (Appendix 13, 1961) which assumes protein-free metabolism:

$$f_{carb} = 1 - [38*(1 - RER)]/[(59.5*RER) - 30.9] \quad (2)$$

The assumption of protein-free metabolism in this equation is likely not accurate. However, there is no waste nitrogen expelled during torpor to allow determination of the amount of protein catabolized. Also, RER values alone cannot disentangle protein from mixed fuel use. Thus, we used this equation to approximate nonlipid fuel use in a way that is directly comparable to estimates by Buck and Barnes (2000), and the value of f_{carb} should reflect a minimum estimate of nonlipid fuel use as catabolism of protein should increase the value of f_{carb}.

3. Results

Metabolic rate of arctic ground squirrels during steady-state torpor increased linearly as T_a decreased from − 2 to − 26 °C (Fig. 1a; reported in Richter et al., 2015). RER of these animals also varied significantly with decreasing T_a in each environmental chamber (Chamber 1: $F_{9,68} = 11.81$, $p < 0.0001$; Chamber 2: $F_{4,34} = 9.07$, $p < 0.0001$; Fig. 1a), but it did not follow a linear pattern. Instead, RER ranged from a maximum mean of 0.76 at − 2 °C to a minimum mean of 0.73 at − 10 °C during steady-state torpor. As T_a decreased below − 10 °C, RER increased until a peak around − 20 °C, after which RER decreased with the continued decrease in T_a. The percentage of carbohydrate fuels utilized, as calculated by the Kleibler equation (Eq. (2)), ranged from a minimum of 18% at − 10 °C to a maximum of 37%

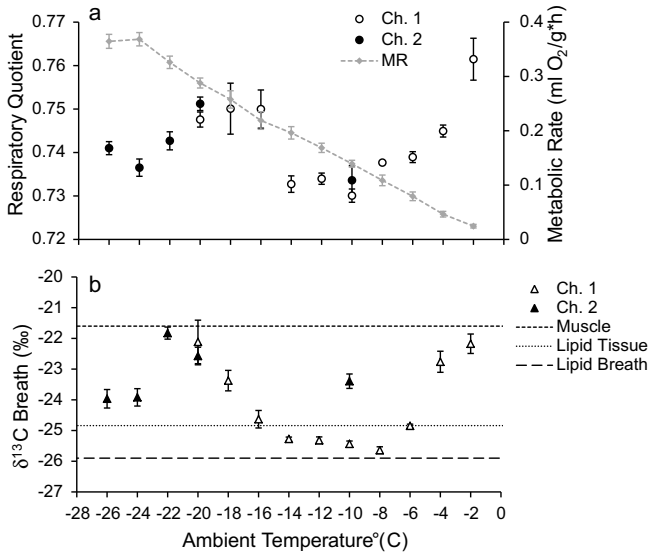

Fig. 1. Respiratory exchange ratio, metabolic rate (a), and $\delta^{13}C$ values of expired breath (b) from torpid arctic ground squirrels repeatedly measured at different ambient temperatures. Squirrels in different environmental chambers experienced different cooling regimes and are represented by different fill (Ch. 1, open; Ch. 2, filled). In (a), respiratory exchange ratio (circles) is plotted with metabolic rate (MR; diamonds) during steady-state torpor; metabolic rate data were combined from both chambers as there were no significant differences between chambers. In (b), the top dotted line (Muscle) represents the $\delta^{13}C$ values of lipid-extracted muscle from hibernating arctic ground squirrels while the middle dotted line (Lipid Tissue) represents $\delta^{13}C$ values from white adipose tissues of hibernating arctic ground squirrels. The lowest dotted line (Lipid Breath) represents the predicted breath $\delta^{13}C$ value from pure lipid catabolism based on lipid tissue $\delta^{13}C$ values adjusted down by 1.1‰ as the potential discrimination between endogenous lipid and breath CO_2. Error bars represent ± SE. Metabolic rate data has been adapted from Richter et al. (2015); $\delta^{13}C$ values of tissues are from Lee et al. (2012).

at − 2 °C.

Breath $\delta^{13}C$ values from torpid arctic ground squirrels followed a similar, nonlinear pattern as RER (Fig. 1b). Mean $\delta^{13}C$ values in squirrel breath at each T_a decreased with decreasing T_a to a minimum of − 25.6‰ at − 8 °C and then increased to a maximum of − 21.8‰ at − 22 °C, then decreased again as T_a decreased further. Breath $\delta^{13}C$ values varied significantly with T_a within each chamber (Chamber 1: $F_{9,68} = 28.78$, $p < 0.0001$; Chamber 2: $F_{4,34} = 15.24$, $p < 0.0001$, Fig. 1b). However, breath $\delta^{13}C$ values were higher at − 10 °C in chamber 2, where squirrels experienced a 10 °C drop in T_a during an arousal episode, compared to chamber 1, where squirrels experienced T_a decreasing in increments of only 2 °C. Breath $\delta^{13}C$ values were similar at − 20 °C, the only other T_a where both chambers were measured (Fig. 1b). The breath $\delta^{13}C$ values and RER of torpid arctic ground squirrels displayed a similar pattern along decreasing T_a (Fig. 1); we found that breath $\delta^{13}C$ values and RER were significantly correlated over the range of values recorded in this study, although RER explained only about a third of the variation in breath $\delta^{13}C$ values ($F_{1,103.5} = 36.97$, $R^2_{adj} = 0.34$, $p < 0.0001$, Fig. 2).

The lower range of $\delta^{13}C$ breath values that we obtained in the present study did not fall between the $\delta^{13}C$ values of lipid-free muscle and white adipose tissue previously obtained from squirrels maintained on the same rodent chow diet: the $\delta^{13}C$ values of breath at T_a − 6 °C to − 14 °C were lower than the $\delta^{13}C$ value of white adipose tissue (Fig. 1b). Using the measured relationship between RER and breath $\delta^{13}C$ values in this study, and the RER value of pure lipid catabolism (0.707, Kleiber, 1961), we calculated a breath $\delta^{13}C$ value for arctic ground squirrels indicative of pure lipid catabolism. This value, − 25.9‰, is 1.1‰ lower than the mean $\delta^{13}C$ value of white adipose tissue in arctic ground squirrels (Lee et al., 2012).

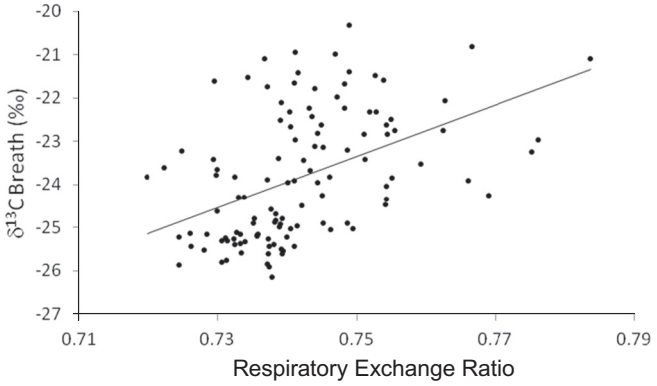

Fig. 2. Relationship between respiratory exchange ratio (RER) and breath $\delta^{13}C$ values in arctic ground squirrels in steady-state torpor at ambient temperatures from − 2 to − 26 °C (n = 17 repeatedly measured; $R^2_{adj} = 0.34$, $p < 0.0001$).

4. Discussion

Carbon isotope ratios of breath CO_2 during torpor varied widely between the endpoints of lipid and lipid-free tissue from hibernating arctic ground squirrels. RER and breath $\delta^{13}C$ values were correlated during steady-state torpor over T_a from − 2 to − 26 °C and a corresponding range of torpid metabolic rates from 0.02 to 0.37 ml O^2/(g ∗ hr) (Richter et al., 2015). This suggests that breath $\delta^{13}C$ values may be a useful tool to investigate endogenous fuel use due to the distinct $\delta^{13}C$ values between lipid and nonlipid fuels. In contrast to the findings of Buck and Barnes (2000) that showed a substantial linear increase in RER with decreasing T_a, we found that temperature effects on RER during torpor were nonlinear and relatively small, and therefore, RER did not span the range needed to further resolve fuel use with the addition of breath $\delta^{13}C$ values.

4.1. Respiratory exchange ratio and breath $\delta^{13}C$ values

This is the first study to document a correlation between RER and breath $\delta^{13}C$ values during metabolism of purely endogenous stores in non-exercising animals. Other animal studies have used endogenous $\delta^{13}C$ signatures and RQ to quantify a shift to isotopically distinct exogenous fuel (Welch et al., 2006, 2008). Our results are in agreement with results from a human study that found a correlation between RER and breath $\delta^{13}C$ values during a shift in endogenous fuel use caused by increasing intensity of exercise (Gautier et al., 1996). RER in the current study only explained about a third of the variation in breath $\delta^{13}C$ values, similar to that measured in exercising humans (~43%, Gautier et al., 1996). This could be due in part to the fact that proteins and carbohydrates do not have consistent $\delta^{13}C$ values relative to lipid. Fasted rats demonstrated $\delta^{13}C$ values of plasma proteins that were intermediate between plasma glucose and lipid, though closer to glucose, while human plasma glucose was intermediate between plasma proteins and lipids (Schoeller et al., 1984). Therefore, while lipid is the minimal endpoint on both the RER and $\delta^{13}C$ scales, the correlation in animals may break down with a shift toward the other macronutrients if carbohydrate is not the maximal endpoint of both scales.

4.2. Isotopic discrimination

Some breath $\delta^{13}C$ values were lower than endogenous lipid values (Fig. 2). The correlation between RER and breath $\delta^{13}C$ values predicts that pure lipid catabolism during hibernation in arctic ground squirrels should result in a breath $\delta^{13}C$ value 1.1‰ lower than that of white adipose tissue (Lee et al., 2012). This observed lipid to breath discrimination during metabolism suggests one of two possible explanations. The first is that lipids bearing the light isotope are preferen-

tially used in metabolism. The second is that ^{13}C is preferentially retained in the circulating bicarbonate pool due to the equilibrium fractionation between bicarbonate and CO_2, causing disproportionate loss of ^{12}C in exhaled CO_2 (Mook et al., 1974; Panteleev et al., 1999; Tabiri et al., 2002). Our data also suggest that endogenous nutrient to breath discrimination factors may be different for lipids, proteins, and carbohydrates: when we adjusted mean $\delta^{13}C$ values of four lipid-extracted skeletal muscles (−21.6‰) down by 1.1‰ (suggested for lipids), some of the breath $\delta^{13}C$ values exceeded the expected value for purely protein or carbohydrate metabolism. Other work has also shown a range in discrimination factors between breath and macronutrients in tissues and diet (Podlesak et al., 2005; Schoeller et al., 1984; Voigt et al., 2008a). While we have only considered 'whole fuel' oxidation, it should also be noted that specific macronutrients may be utilized differentially (e.g., selective mobilization of fatty acids, reviewed in Price and Valencak, 2012) and these specific macronutrients are likely to differ in $\delta^{13}C$ values (Budge et al., 2011; Larsen et al., 2009). All of these considerations illustrate the need for further work to define the discrimination factors in specific pathways, especially among the catabolic pathways of various fuel sources, as the use of breath $\delta^{13}C$ values is further developed.

4.3. Breath $\delta^{13}C$ values and metabolic intensity

In torpid arctic ground squirrels, metabolic rate increases linearly with an increase in thermal challenge (Buck and Barnes, 2000; Richter et al., 2015). In the current study, a decrease in T_a from −2 to −26 °C invoked a 15-fold increase in torpid metabolic rate from 0.02 to 0.37 ml $O^2/(g * hr)$ (Richter et al., 2015). The breath $\delta^{13}C$ values did not track the increase in torpid metabolic rate, but instead remained correlated with RER (Fig. 1). Human studies have found an increase in breath $\delta^{13}C$ values with increasing exercise intensity (Gautier et al., 1996; McCue et al., 2015), indicating use of nonlipid fuel. These studies further support that $\delta^{13}C$ values of breath are indicative of metabolized fuel, which changes as a result of physiological conditions (i.e., exercising, fasting, etc.), instead of responding only to an increase in metabolism.

4.4. Potential factors affecting fuel selection

A previous investigation of metabolism of arctic ground squirrels during torpor reported a significant, linear increase in RER with decreasing T_a below 0 °C (Buck and Barnes, 2000), suggestive of a 76% reliance on nonlipid fuels at a T_a of −16 °C compared to only 28% at a T_a of 0 °C. In the current study, RER suggested a maximum reliance on nonlipid fuels of 37% at a T_a of −2 °C and a minimum of 18% at a T_a of −10 °C, followed by an increase at T_a below −10 °C. We are unsure why animals in the current study did not exhibit a similar increase in RER with decreased T_a. We cannot exclude the possibility that differences could be due to improved methodology available in the current experiments: the current system had a better drying method for gas without CO_2 adsorption and also software compensating for both baseline and span drift, while only a fixed baseline value was available in the previous study. However, the differences between studies might also relate to differences in body composition of the study animals. The animals in the previous study were sampled over different years and at different times during a season, while the animals in the current study were sampled repeatedly within the same year and sequentially across the first few months of the hibernation season. Animals in the previous study may have been measured at low lipid masses, making them more reliant on nonlipid sources: natural fasters will increasingly rely on nonlipid fuels as the duration of the fast increases and adiposity decreases (Caloin, 2004; Cherel et al., 1993, 1992). Animals in the current study would have had the largest lipid reserves at the beginning and sequentially depleted the reserves throughout the relatively short term of the study as T_a also decreased. Thus, temperature effects on metabolic fuel selection may have been confounded by differences in adiposity, as it also influences the ratio of lipid to protein utilization (Caloin, 2004).

Another factor contributing to the differences in fuel use between the current study and that of Buck and Barnes (2000) may be the physiological adjustments needed for the larger changes in T_a (4 °C in previous study vs. 2 °C in current). This might have increased reliance on nonlipid fuels in a manner similar to strenuous exercise before training causing increased carbohydrate use (Holloszy et al., 1998). In support of this, squirrels experiencing a 10 °C change to T_a of −10 °C in the current study had more variable RER and higher $\delta^{13}C$ signatures compared with squirrels experiencing a 2 °C change. Differences in the magnitude of T_a change could help explain the lesser response seen in the current study, while body composition may account for why the shift toward nonlipid fuel use occurred at a T_a lower than in the previous study of Buck and Barnes (2000).

5. Conclusion

This study answers a call to expand the use of ^{13}C-breath testing into new research areas (McCue and Welch, 2016; Welch et al., 2016) and demonstrates that breath $\delta^{13}C$ values can be used to identify metabolic shifts between endogenous substrates with naturally distinct $\delta^{13}C$ signatures in animals. The correlation between $\delta^{13}C$ values in breath and RER provides a foundation for using breath $\delta^{13}C$ values as an index of endogenous fuel use. The breath $\delta^{13}C$ value extrapolated from our data to coincide with pure lipid metabolism was 1.1‰ more depleted than lipid stores, which suggests discrimination may occur during the catabolism of endogenous fuel stores. Further work is needed to confirm this and delineate discrimination factors resulting from metabolic pathways between endogenous substrates and respired CO_2.

Acknowledgments

We thank Tim Stevenson, Brady Salli, and Jermey Becker for help with captive animal care and Franziska Kohl for animal and logistics support. We also thank Tim Howe and Norma Haubenstock of the Alaska Stable Isotope Facility for support in sample preparation and timely analysis. We thank two anonymous reviewers for thoughtful comments that improved this manuscript. This work was supported by a collaborative National Science Foundation International Polar Year Grant [grant numbers EF-0732755 to BMB and DMO, EF-0732763 to CLB], a National Science Foundation Grant [grant number 158160 to BMB], and a United States Army Medical Research and Materiel Command Grant to BMB [grant number 05178001].

References

Barnes, B.M., 1989. Freeze avoidance in a mammal: body temperatures below 0 °C in an arctic hibernator. Science 244 (4912), 1593–1595.

Berg, J.M., Tymoczko, J.L., Stryer, L., 2002. Biochemistry, fifth ed. W H Freeman, New York.

Bickler, P.E., 1984. CO_2 balance of a heterothermic rodent: comparison of sleep, torpor, and awake states. Am. J. Phys. 246, R49–R55.

Boonstra, R., Bradley, A.J., Delehanty, B., 2011. Preparing for hibernation in ground squirrels: adrenal androgen production in summer linked to environmental severity in winter. Funct. Ecol. 25 (6), 1348–1359. http://dx.doi.org/10.1111/j.1365-2435.2011.01890.x.

Buck, C.L., Barnes, B.M., 1999a. Annual cycle of body composition and hibernation in free-living arctic ground squirrels. J. Mammal. 80 (2), 430–442.

Buck, C.L., Barnes, B.M., 1999b. Temperatures of hibernacula and changes in body composition of arctic ground squirrels over winter. J. Mammal. 80 (4), 1264–1276.

Buck, C.L., Barnes, B.M., 2000. Effects of ambient temperature on metabolic rate, respiratory quotient, and torpor in an arctic hibernator. Am. J. Physiol. Regul. Integr. Comp. Physiol. 279 (1), R255–R262.

Buck, C.L., Breton, A., Kohl, F., Tøien, Ø., Barnes, B.M., 2008. Overwinter body temperature patterns in free-living Arctic squirrels (*Spermophilus parryii*). In: Lovegrove, B.G., McKechnie, A.E. (Eds.), Hypometabolism in Animals: Hibernation, Torpor and Cryobiology. University of KwaZulu-Natal, Pietermaritzburg, pp. 317–326.

Budge, S.M., Wang, S.W., Hollmen, T.E., Wooller, M.J., 2011. Carbon isotopic fractionation in eider adipose tissue varies with fatty acid structure: implications for

trophic studies. J. Exp. Biol. 214 (3790–380).
Caloin, M., 2004. Modeling of lipid and protein depletion during total starvation. Am. J. Physiol. Endocrinol. Metab. 287 (4), E790–E798. http://dx.doi.org/10.1152/ajpendo.00414.2003.
Cannon, B., Nedergaard, J., 1979. The physiological role of pyruvate carboxylation in hamster brown adipose tissue. Eur. J. Biochem. 94, 419–426.
Cherel, Y., Robin, J.-P., Heitz, A., Calgari, C., Lemaho, Y., 1992. Relationships between lipid availability and protein utilization during prolonged fasting. J. Comp. Physiol. B. 162 (4), 305–313.
Cherel, Y., Fréby, F., Gilles, J., Robin, J.-P., 1993. Comparative fuel metabolism in Gentoo and king penguins: adaptation to brief versus prolonged fasting. Polar Biol. 13 (4), 263–269.
Dark, J., 2005. Annual lipid cycles in hibernators: integration of physiology and behavior. Annu. Rev. Nutr. 25, 469–497.
DeNiro, M.J., Epstein, S., 1977. Mechanism of carbon isotope fractionation associated with lipid synthesis. Science 197 (4300), 261–263.
Galster, W., Morrison, P.R., 1975. Gluconeogenesis in arctic ground squirrels between periods of hibernation. Am. J. Phys. 228 (1), 325–330.
Gautier, J.F., Pirnay, F., Lacroix, M., Mosora, F., Scheen, A.J., Cathelineau, G., Lefebvre, P.J., 1996. Changes in breath $^{13}CO_2/^{12}CO_2$ during exercise at different intensities. J. Appl. Physiol. 81 (3), 1096–1102.
Haldane, J.S., 1912. Methods of Air Analysis. Griffin, London.
Hatch, K.A., Pinshow, B., Speakman, J.R., 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 (1), 21–33.
Holloszy, J.O., Kohrt, W.M., Hansen, P.A., 1998. The regulation of carbohydrate and fat metabolism during and after exercise. Front. Biosci. 3, d1011–d1027.
Karpovich, S.A., Tøien, Ø., Buck, C.L., Barnes, B.M., 2009. Energetics of arousal episodes in hibernating arctic ground squirrels. J. Comp. Physiol. B. 179 (6), 691–700. http://dx.doi.org/10.1007/s00360-009-0350-8.
Kleiber, M., 1961. The Fire of Life: An Introduction to Animal Energetics. John Wiley and Sons, New York.
Krilowicz, B.L., 1985. Ketone body metabolism in a ground squirrel during hibernation and fasting. Am. J. Phys. 249 (4), R462–R470.
Larsen, T., Taylor, D.L., Leigh, M.B., O'Brien, D.M., 2009. Stable isotope fingerprinting: a novel method for identifying plant, fungal, or bacterial origins of amino acids. Ecology 90 (12), 3526–3535.
Lee, T.N., Buck, C.L., Barnes, B.M., O'Brien, D.M., 2012. A test of alternative models for increased tissue nitrogen isotope ratios during fasting in hibernating arctic ground squirrels. J. Exp. Biol. 215, 3354–3361. http://dx.doi.org/10.1242/jeb.068528.
Luft, U.C., Myhre, L.G., Loeppky, J.A., 1973. Validity of Haldane calculation for estimating respiratory gas exchange. J. Appl. Physiol. 34 (6), 864–865.
McCue, M.D., Pollock, E.D., 2013. Measurements of substrate oxidation using $^{13}CO_2$-breath testing reveals shifts in fuel mix during starvation. J. Comp. Physiol. B. 183, 1039–1052. http://dx.doi.org/10.1007/s00360-013-0774-z.
McCue, M.D., Welch, K.C., 2016. ^{13}C-breath testing in animals: theory, applications, and future directions. J. Comp. Physiol. B. 186 (3), 265–285. http://dx.doi.org/10.1007/s00360-015-0950-4.
McCue, M.D., Passement, C.A., Rodriguez, M., 2015. 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. A 179, 164–171. http://dx.doi.org/10.1016/j.cbpa.2014.08.021.
McWilliams, S.R., Guglielmo, C., Pierce, B., Klaassen, M., 2004. Flying, fasting, and feeding in birds during migration: a nutritional and physiological ecology perspective. J. Avian Biol. 35 (5), 377–393. http://dx.doi.org/10.1111/j.0908-8857.2004.03378.x.
Mook, W.G., Bommerson, J.C., Staverman, W.H., 1974. Carbon isotope fractionation between dissolved bicarbonate and gaseous carbon dioxide. Earth Planet. Sci. Lett. 22, 169–176.
Owen, O.E., Kalhan, S.C., Hanson, R.W., 2002. The key role of anaplerosis and cataplerosis for citric acid cycle function. J. Biol. Chem. 277 (34), 30409–30412. http://dx.doi.org/10.1074/jbc.R200006200.
Panteleev, N., Peronnet, F., Hillaire-Marcel, C., Lavoie, C., Massicotte, D., 1999. Carbon isotope fractionation between blood and expired CO_2 at rest and exercise. Respir. Physiol. 116, 77–83. http://dx.doi.org/10.1016/S0034-5687(99)00027-4.
Pataki, D.E., 2005. Emerging topics in stable isotope ecology: are there isotope effects in plant respiration? New Phytol. 167 (2), 321–323. http://dx.doi.org/10.1111/j.1469-8137.2005.01498.x.
Perkins, S.E., Speakman, J.R., 2001. Measuring natural abundance of ^{13}C in respired CO_2: variability and implications for non-invasive dietary analysis. Funct. Ecol. 15 (6), 791–797. http://dx.doi.org/10.1046/j.0269-8463.2001.00577.x.
Podlesak, D.W., McWilliams, S.R., Hatch, K.A., 2005. Stable isotopes in breath, blood, feces and feathers can indicate intra-individual changes in the diet of migratory songbirds. Oecologia 142 (4), 501–510. http://dx.doi.org/10.1007/s00442-004-1737-6.
Price, E.R., Valencak, T.G., 2012. Changes in fatty acid composition during starvation in vertebrates: mechanisms and questions. In: McCue, M.D. (Ed.), Comparative Physiology of Fasting, Starvation, and Food Limitation. Springer, New York, pp. 237–255.
Richter, M.M., Williams, C.T., Lee, T.N., Tøien, Ø., Florant, G.L., Barnes, B.M., Buck, C.L., 2015. Thermogenic capacity at subzero temperatures: how low can a hibernator go? Physiol. Biochem. Zool. 88 (1), 81–89. http://dx.doi.org/10.1086/679591.
Robbins, C.T., 2001. Wildlife Feeding and Nutrition, second ed. Academic Press, San Diego.
Schoeller, D.A., Brown, C., Nakamura, K., Nakagawa, A., Mazzeo, R.S., Brooks, G.A., Budinger, T.F., 1984. Influence of metabolic fuel on the $^{13}C/^{12}C$ ratio of breath CO_2. Biomed. Mass Spectrom. 11 (11), 557–561. http://dx.doi.org/10.1002/bms.1200111103.
Sheriff, M.J., Fridinger, R.W., Tøien, Ø., Barnes, B.M., Buck, C.L., 2013. Metabolic rate and prehibernation fattening in free-living arctic ground squirrels. Physiol. Biochem. Zool. 86 (5), 515–527.
Snapp, B.D., Heller, H.C., 1981. Suppression of metabolism during hibernation in ground squirrels (*Citellus lateralis*). Physiol. Zool. 54, 297–307.
Staples, J.F., Hochachka, P.W., 1998. The effect of hibernation status and cold-acclimation on hepatocyte gluconeogenesis in the golden-mantled ground squirrel (*Spermophilus lateralis*). Can. J. Zool. 76 (9), 1734–1740. http://dx.doi.org/10.1139/cjz-76-9-1734.
Tabiri, H.Y., Bertolo, F.P., Ball, R.O., Korver, D.R., 2002. Development of the indicator amino acid oxidation technique in chickens: calibration of oxidation system and determination of bicarbonate retention factor. Poult. Sci. 81, 1020–1025.
Tcherkez, G., Nogués, S., Bleton, J., Cornic, G., Badeck, F., Ghashghaie, J., 2003. Metabolic origin of carbon isotope composition of leaf dark-respired CO_2 in French bean. Plant Physiol. 131 (1), 237–244. http://dx.doi.org/10.1104/pp.013078.
Tøien, Ø., 2013. Automated open flow respirometry in continuous and long-term measurements: design and principles. J. Appl. Physiol. 114, 1094–1107.
Vallerand, A.L., Pérusse, F., Bukowiecki, L.J., 1990. Stimulatory effects of cold exposure and cold acclimation on glucose uptake in rat peripheral tissues. Am. J. Phys. 259 (5), R1043–R1049.
Vock, R., Hoppeler, H., Claassen, H., Wu, D.X.Y., Billeter, R., Weber, J.-M., Taylor, C.R., Weibel, E.R., 1996. Design of the oxygen and substrate pathways. VI. Structural basis of intracellular substrate supply to mitochondria in muscle cells. J. Exp. Biol. 199, 1689–1697.
Voigt, C.C., Baier, L., Speakman, J.R., Siemers, B.M., 2008a. Stable carbon isotopes in exhaled breath as tracers for dietary information in birds and mammals. J. Exp. Biol. 211 (14), 2233–2238. http://dx.doi.org/10.1242/jeb.018523.
Voigt, C.C., Grasse, P., Rex, K., Hetz, S.K., Speakman, J.R., 2008b. Bat breath reveals metabolic substrate use in free-ranging vampires. J. Comp. Physiol. B. 178 (1), 9–16. http://dx.doi.org/10.1007/s00360-007-0194-z.
Wagner, J.A., Horwarth, S.M., Dahms, T.E., Reed, S., 1973. Validation of open-circuit method for the determination of oxygen consumption. J. Appl. Physiol. 34, 859–863.
Wang, L.C.H., Wolowyk, M.W., 1988. Torpor in mammals and birds. Can. J. Zool. 66 (1), 133–137.
Welch, K.C., Bakken, B.H., Martínez del Rio, C., Suarez, R.K., 2006. Hummingbirds fuel hovering flight with newly ingested sugar. Physiol. Biochem. Zool. 79 (6), 1082–1087. http://dx.doi.org/10.1086/507665.
Welch, K.C., Herrera, L.G., Suarez, R.K., 2008. Dietary sugar as a direct fuel for flight in the nectarivorous bat *Glossophaga soricina*. J. Exp. Biol. 211 (3), 310–316. http://dx.doi.org/10.1242/jeb.012252.
Welch, K.C., Péronnet, F., Hatch, K.A., Voigt, C.C., McCue, M.D., 2016. Carbon stable-isotope tracking in breath for comparative studies of fuel use. Ann. N.Y. Acad. Sci. 1365, 15–32. http://dx.doi.org/10.1111/nyas.12737.
Williams, C.T., Goropashnaya, A.V., Buck, C.L., Fedorov, V.B., Kohl, F., Lee, T.N., Barnes, B.M., 2011. Hibernating above the permafrost: effects of ambient temperature and season on expression of metabolic genes in liver and brown adipose tissue of arctic ground squirrels. J. Exp. Biol. 214, 1300–1306. http://dx.doi.org/10.1242/jeb.052159.