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Carbon isotopes in exhaled breath track metabolic substrates in brown bears (*Ursus arctos*)

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Dietary carbon is oxidized and exhaled as CO_2, thus $\delta^{13}C_{breath}$ values can provide information on diet and substrate use for energy. However, physiological phenomena such as fat deposition and fasting can alter values of $\delta^{13}C_{breath}$ such that interpretation of source contributions may be unclear. Consequently, before application to free-ranging animals, inferences about feeding and nutritional states based on $\delta^{13}C_{breath}$ should be validated with controlled experiments using captive individuals. Here, we report $\delta^{13}C_{breath}$ values for 4 captive brown bears (*Ursus arctos*) under different conditions: the bears were 1st given a diet containing carbohydrate, lipid, and protein; they were then switched to a carbohydrate-free diet consisting of salmon and fish oil; and finally they were placed on a fast leading to winter hibernation. Following the switch to the carbohydrate-free diet, values of $\delta^{13}C_{breath}$ and $\delta^{13}C_{plasma}$ suggested that although oxidation included a substantial portion of dietary proteins, dietary lipids were preferentially utilized. After the onset of fasting, $\delta^{13}C_{breath}$ values did not change, possibly because of selective mobilization of endogenous fatty acids accreted by bears while consuming the experimental diet. Examination of our data suggests that because CO_2 production and exhalation are influenced differentially by fasting state and diet composition, additional measurements such as respiratory exchange ratio should be used to aid interpretation of carbon isotope analysis of breath.

Key words: breath, brown bear, CO_2, diet, fasting, RER, respiratory exchange ratio, stable isotope, *Ursus arctos*

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Physiological processes such as assimilation of dietary nutrients (carbohydrate, lipid, and protein) and fasting affect stable isotope ratios in animal tissues, allowing researchers to infer diet composition, foraging strategies, and nutritional status from tissue analyses (Martínez del Rio et al. 2009). Inferences also can be drawn from the $\delta^{13}C$ value of exhaled CO_2; although common in human medical practices, this has only recently been applied to animal ecology (Hatch et al. 2002).

Measurements of ^{13}C in breath following an injection of isotopically labeled bicarbonate may be used to calculate energetic expenditure (Ben-David and Flaherty 2012; Speakman and Thomson 1997), but thus far the most common application has been to infer patterns of oxidation of different nutrients for energy. For example, researchers measuring $\delta^{13}C_{breath}$ values concluded that the high energetic demand of flight in birds and bats is generally met by immediate oxidation of ingested food, rather than endogenous energy stores (Voigt et al. 2010; Welch et al. 2006). In contrast, $\delta^{13}C_{breath}$ analysis of free-ranging polar bears (*Ursus maritimus*) during summer on shore in western Hudson Bay, Canada, suggested that although these animals consumed substantial amounts of vegetation, they primarily oxidized stored lipids (Hobson et al. 2009).

Dietary carbohydrate, lipid, and protein can be oxidized, primarily in the tricarboxylic acid cycle, during which carbon atoms are cleaved from these nutrients and released as CO_2 (Fig. 1). The arrangement of carbon atoms and their oxidation states differ among nutrients, thus the energy released and the

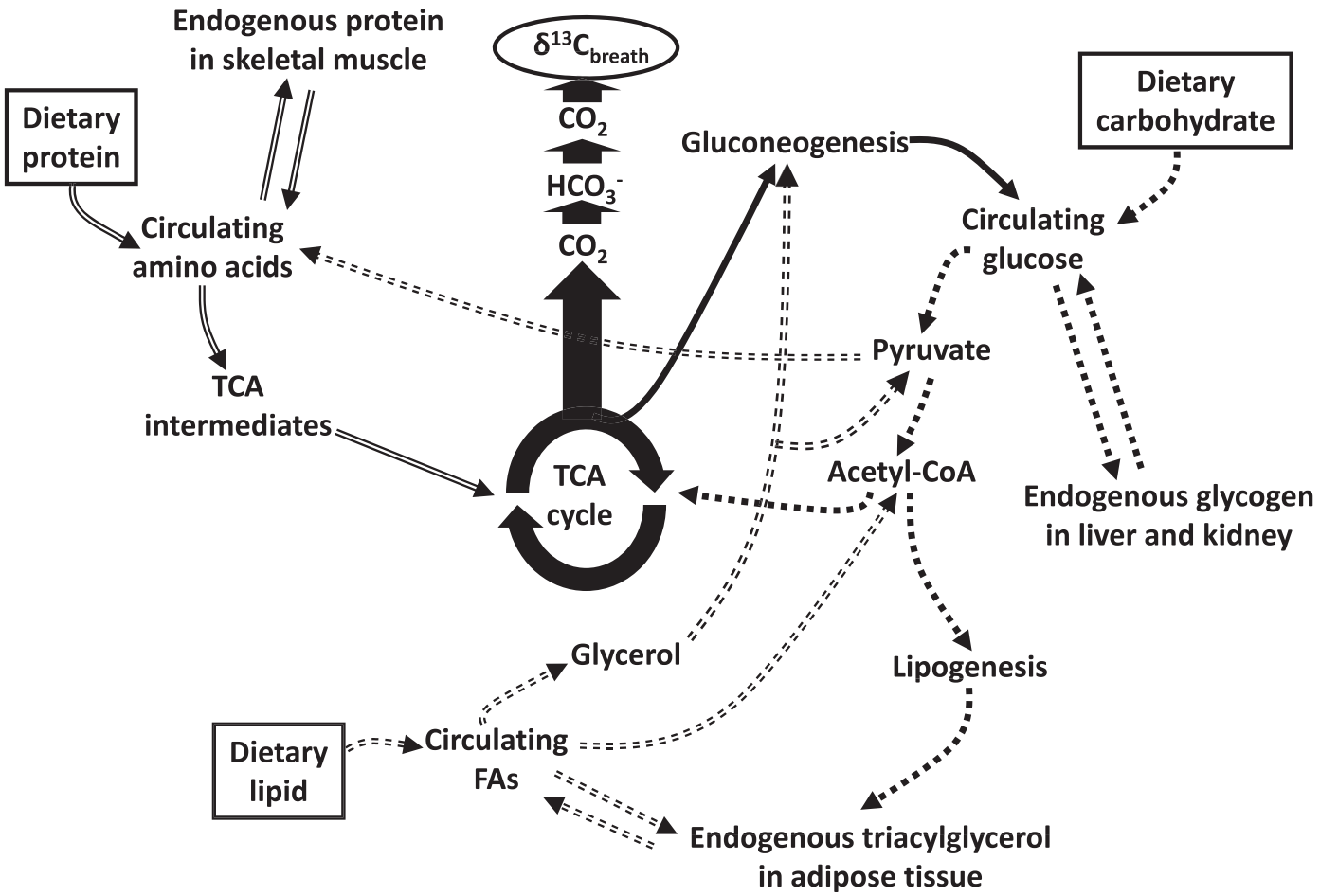

Fig. 1.—Major carbon metabolism pathways of dietary protein (double, solid lines), dietary lipid (double, dashed lines; note that ''FAs'' are nonesterified fatty acids), and dietary carbohydrate (single, dashed lines) in mammals. Each nutrient can be immediately oxidized for energy or used to synthesize endogenous tissue. The heavy black circle at the center is the tricarboxylic acid cycle, which is the main source of CO_2 production for mammals. The CO_2 is converted to bicarbonate (HCO_3^-) for vascular transport to the lungs, where it is converted back to CO_2 and exhaled. Thus, carbon from nutrients oxidized in the tricarboxylic acid cycle, both dietary and endogenous, create $\delta^{13}C_{breath}$ values. CO_2 also is generated by pathways not shown here but, in most situations, such other fluxes are minor and have negligible influence on $\delta^{13}C_{breath}$ values. Ketogenesis is not shown.

reaction stoichiometry differ as well. Indeed, the ratio of CO_2 produced to O_2 consumed in a given period of time (the respiratory exchange ratio [RER]) reflects the type of nutrient oxidized: values of 1.00 indicate glucose oxidation, whereas values of approximately 0.70 indicate lipid oxidation. Values of approximately 0.80 indicate oxidation of protein or of a combination of 2 or 3 nutrient types (Brody 1999). After CO_2 is produced by the tricarboxylic acid cycle, most of it is converted to bicarbonate (HCO_3^-) in the blood, which is circulated to the lungs, where CO_2 is reformed and exhaled (Fig. 1).

Nutrients also can be routed to anabolic processes rather than oxidation. Proteins are critical components of tissues that perform important enzymatic, mechanical, and structural roles (e.g., skeletal muscle—Brody 1999), and it is expected that most dietary proteins are routed to biosynthesis (Voigt et al. 2008b). Lipids may be used for biosynthesis (e.g., adipose tissue) or oxidized, whereas in general, carbohydrates are more likely to be oxidized (Tieszen and Fagre 1993; Voigt et al. 2008b). In addition, the amino acids, monosaccharides, and triacylglycerols (glycerol molecules linked to 3 fatty acids [FAs]) that compose nutrients can have unique chemical properties that affect their routing, as well as unique isotopic properties (McCue et al. 2010). Tissue or breath isotopic signatures therefore can reflect routing of these monomers as well as routing of the nutrients.

Free-ranging animals vary their diets and metabolic processes over time, influencing the fates of dietary carbon and $\delta^{13}C_{breath}$ values. An animal consuming all 3 nutrient types will either oxidize the monomers or use them to resynthesize glycogen, triacylglycerol, and protein (Fig. 1; Brody 1999). Even on a nutrient-sufficient diet, a small amount of each endogenous nutrient is oxidized during the natural degradation and replacement (i.e., turnover) of tissue. When oxidative nutrients shift (e.g., after a diet switch or the onset of fasting, as described below), values of $\delta^{13}C_{breath}$ should reflect the new carbon source for CO_2 in less than a day (Ayliffe et al. 2004; Sponheimer et al. 2006). In contrast, $\delta^{13}C_{plasma}$ generally reflects carbon input from a longer time span (e.g., the previous 10 days in black bears [*Ursus*

Table 1.—Treatments, sampling schedule, and diet items of captive brown bears switched from a summer diet to an experimental diet then fasted for winter hibernation. RER = respiratory exchange ratio.

	Summer diet	Experimental diet		Hibernation
Dates	1 March–14 September	15 September–26 October		27 October–1 March
Diet			$\delta^{13}C$	Fasting
	Dog food	Salmon[a]	−21.6	
	Apples	Salmon LE[a,b]	−19.4	
	Pastries	Fish oil[a]	−25.5	
Sampling occasions	Day 0 (14 September)	Day 28 (12 October)		Day 57 (10 November)
		Day 42 (26 October)		Day 70 (23 November)
				Day 168 (1 March)
Samples	Breath	Breath		Breath
	Plasma	Plasma		Plasma (November only)
	RER			RER (1 bear, 23 November)

[a] Diet was 0.87 salmon and 0.13 fish oil by fresh weight.
[b] LE = lipid-extracted.

americanus—Hilderbrand et al. 1996]). Dietary carbon requires more time to appear in plasma because degradation of old tissue and synthesis of new tissue are slower processes than oxidation.

An animal consuming a low-carbohydrate diet likely oxidizes lipids primarily, because they contain approximately twice as many joules per gram than protein (Brody 1999). However, animals also must maintain blood glucose because brain cells cannot oxidize FAs (Morris 2005). Glucose can be released into circulation from liver glycogen or newly synthesized by gluconeogenesis, using amino acids incorporated into the tricarboxylic acid cycle, or glycerol (Fig. 1). Alternatively, the brain can oxidize ketone bodies, which also are synthesized from amino acids and FAs (Brody 1999; Morris 2005). Subsequent oxidation of newly formed glucose and ketone bodies yields CO_2 containing carbon atoms from the amino acid, FA, or glycerol precursors. Thus, in animals consuming low-carbohydrate diets, $\delta^{13}C_{breath}$ values likely reflect dietary lipid, or a combination of lipids and protein, if the latter is heavily used for gluconeogenesis or ketogenesis.

A fasting animal generally depletes liver glycogen in less than a day (Shreeve 1974; but see McCue 2010), placing more importance on gluconeogenesis. However, the mobilization of endogenous amino acids for gluconeogenesis is minimized because this can damage tissue and hinder organ function (Brody 1999; Lohuis et al. 2007). As a result, stored lipids become the main metabolic fuel during a fast, and values of $\delta^{13}C_{breath}$ should largely reflect endogenous lipid.

Because diet composition and fasting have complex influences on CO_2 production and $\delta^{13}C_{breath}$ values, controlled feeding studies on captive animals are necessary for interpreting $\delta^{13}C_{breath}$ data collected from free-ranging individuals (Voigt et al. 2008a). In this study 4 captive brown bears (*Ursus arctos*) were fed a summer diet of carbohydrate, lipid, and protein for several months then switched to a carbohydrate-free diet (Table 1). Six weeks after the diet shift, bears were fasted and then entered winter hibernation (hereafter referred to as fasting). Blood and exhaled breath were sampled coincident with the diet switch and periodically thereafter.

We predicted that while bears were consuming the experimental diet, $\delta^{13}C_{breath}$ values would reflect preferential oxidation of dietary lipids because of their high energy content and the routing of dietary protein to biosynthesis. In that situation, exhaled CO_2 should primarily include carbon from dietary lipids, whereas plasma should include carbon from dietary proteins consumed over the previous 10 days (Hilderbrand et al. 1996). As a result of this routing we predicted that after the switch from the summer diet to the experimental diet, changes in $\delta^{13}C_{breath}$ values would differ in magnitude from changes in $\delta^{13}C_{plasma}$ values. Lastly, we predicted that after the onset of fasting, $\delta^{13}C_{breath}$ values would change because of the abrupt reliance on endogenous energy stores and then remain constant throughout the fasting period.

Materials and Methods

Sampling of bears.—Two adult male (A and B) and 2 adult female (C and D) brown bears were housed at the Washington State University Bear Research, Education and Conservation Center in Pullman, Washington. Between approximately 1 March and 14 September 2009, bears were offered a summer diet of commercial dog chow, apples, and pastries ad libitum. On 14 September (day 0 of this experiment) bears ranged in body mass from 123 kg to 303 kg, and were switched to an experimental diet consisting of fish oil (hereafter ''oil'') mixed with chinook salmon (*Oncorhynchus tshawytscha*; Table 1). On day 42 (26 October), feeding was discontinued and bears entered hibernation. On day 57, bears A and B were moved to an enclosure kept at approximately 10°C for the remainder of the winter and were visually checked every 2–4 days; bears C and D experienced ambient temperatures and were visually checked monthly.

We blow-darted bears for immobilization and sampling on days 0, 28, 42, 57, 70, and 168 with a mixture of Telazol, ketamine hydrochloride (Fort Dodge Animal Health, Fort Dodge, Iowa), and xylazine hydrochloride (Vedco Inc., St. Joseph, Missouri; Table 1). Ten milliliters of blood was collected from the jugular vein into a heparinized tube. Samples were centrifuged and plasma was siphoned and

frozen. We fitted a canine anesthesia mask (Smiths Medical PM Inc., Norwell, Massachusetts) over the nostrils and lips of each bear and attached a custom 2-way valve (Hans Rudolph Inc., Shawnee, Kansas) to the mask. The bear inhaled fresh air through 1 valve and exhaled breath through the other, which was connected to a 25-liter Douglas bag (Harvard Apparatus, Holliston, Massachusetts) that had been rolled tightly to purge ambient air. Bears breathed freely until the bag was full, then we removed the mask and attached a 30-ml syringe to a luer lock port on the bag. We extracted a subsample of breath and injected it into 2 evacuated vials containing magnesium perchlorate and cotton. Methods used in this research were approved by Institutional Animal Care and Use Committees at University of Wyoming and Washington State University, and followed guidelines of the American Society of Mammalogists (Sikes et al. 2011).

Analyses of breath, plasma, and diet items.—Plasma and diet samples were lyophilized then homogenized prior to analysis. We removed lipids from aliquots of salmon samples by soxhlet extraction for 5 h using a 2:1 chloroform : methanol solvent solution. Approximately 1 mg of sample was combusted in an elemental analyzer (Carlo Erba, Milan, Italy) interfaced to a Micromass Optima mass spectrometer (Micromass United Kingdom Ltd., Manchester, United Kingdom) operated in continuous-flow mode (Fry et al. 1992), at the United States Geological Survey stable isotope laboratory (Denver, Colorado). Isotope values are expressed in δ notation: $\delta^{13}C = (R_{sample}/R_{standard} - 1) \times 1{,}000$, where $\delta^{13}C$ values are reported in parts per thousand (‰) deviation relative to a standard gas and R represents $^{13}C/^{12}C$ for samples and the standard. Isotopic data were normalized to Vienna Peedee Belemnite (VPDB) using the primary standards United States Geological Survey 40 and 41. Analytical error and accuracy were assessed through replicate measures of primary standards and were less than 0.2‰. A secondary standard (reagent-grade keratin) was analyzed in duplicate within each analytical sequence and used as a quality control check; reproducibility was better than 0.2‰.

Breath samples were sent to the University of Wyoming Stable Isotope Facility (Laramie, Wyoming) for carbon isotope analysis within 9 weeks of collection. Breath vials were sampled via an autosampler (PAL; LEAP Technologies, Carrboro, North Carolina) linked to a gas bench (Thermo Finnigan GasBench II; Thermo Electron Corp, Waltham, Massachusetts) interfaced with a continuous flow mass spectrophotometer (Thermo Finnigan Delta^{PLUS} XP Continuous Flow Stable Isotope Ratio Mass Spectrometer; Thermo Electron Corp.). Gas standards used for analysis contained CO_2 of known $\delta^{13}C$ values, which included 2 normalizers (−10.12‰ and −25.36‰) and 1 standard check (−10.96‰).

Because we lacked isotopic values for the summer diet offered prior to our experiment, we estimated nutrient oxidation on day 0 with RER. Breath from the Douglas bag was drawn through a field gas analysis system (Sable Systems FX 0501-20; Sable Systems International, Las Vegas, Nevada) at 200 ml/min and percent O_2 and CO_2 were measured every 2 s for ≥5 min. Measurements were corrected for drift following standard protocols and RER was calculated using equations provided by Sable Systems International. RER was measured and calculated again during the fasting period on day 70.

Calculations and statistical analyses.—We compared values of $\delta^{13}C_{breath}$ among sampling occasions using Friedman's repeated-measures, rank-based analysis of variance, and we made post hoc pairwise comparisons with the Student–Newman–Keuls method (Zar 2010). Normality was checked using the Kolmogorov–Smirnov test (assumptions accepted at $P > 0.050$). We compared $\delta^{13}C_{plasma}$ values among sampling occasions with a 1-way, repeated-measures analysis of variance, and made post hoc comparisons with the Holm–Sidak method (Kersey et al. 2010). Assumptions of normality (Kolmogorov–Smirnov test) and equal variance (Levene's median test) were evaluated. Residual plots were inspected for both $\delta^{13}C_{breath}$ and $\delta^{13}C_{plasma}$.

To assess whether changes in $\delta^{13}C_{breath}$ values differed in magnitude from those in $\delta^{13}C_{plasma}$ values after the switch to the experimental diet, we compared changes in $\delta^{13}C$ values of breath and plasma between day 0 and day 28, and day 0 and day 42, using paired, 2-tailed *t*-tests. Normality was checked with the Kolmogorov–Smirnov test. We set $\alpha = 0.050$ for all comparisons, and statistical analyses were conducted using SigmaStat 3.11 (Systat Software, Inc., Point Richmond, California).

By fresh weight, the experimental diet consisted of 0.87 salmon and 0.13 oil. The oil portion of the diet included 1.00 lipid. The salmon portion included 0.73 water, 0.18 protein, 0.06 lipid, and 0.03 carbohydrates and minerals. For simplicity in isotope calculations and because the carbohydrate and mineral fractions of the salmon were small, we combined them with the protein fraction, yielding values of 0.73 water, 0.21 protein combined with carbohydrate and mineral (hereafter ''salmon protein''), and 0.06 lipid (hereafter ''salmon lipid''). Dry matter composition of this portion was thus 0.78 salmon protein and 0.22 salmon lipid. For the total experimental diet, dry matter composition was thus 0.49 salmon protein, 0.14 salmon lipid, and 0.37 oil. Unfortunately, $\delta^{13}C_{salmon\ lipid}$ was not measured directly; instead, we calculated it based on $\delta^{13}C$ values of whole salmon (which was not lipid-extracted) and lipid-extracted (LE) salmon using the mass balance equation:

$$\delta^{13}C_{salmon} = \left(\delta^{13}C_{salmon\ LE} \times 0.78\right) + \left(\delta^{13}C_{salmon\ lipid} \times 0.22\right), \quad (1)$$

where $\delta^{13}C_{salmon}$ and $\delta^{13}C_{salmon\ LE}$ are known. To estimate contributions of dietary sources to oxidation while bears were consuming the experimental diet, we constructed the mass balance equation:

$$\delta^{13}C_{breath} = \left(\delta^{13}C_{salmon\ LE} \times f_{salmon\ LE}\right) + \left(\delta^{13}C_{salmon\ lipid} \times f_{salmon\ lipid}\right) + \left(\delta^{13}C_{oil} \times f_{oil}\right), \quad (2)$$

where f is the proportional contribution of each source to oxidation and:

$$1 = f_{salmon\ LE} + f_{salmon\ lipid} + f_{oil}. \quad (3)$$

We then calculated predicted $\delta^{13}C_{breath}$ values for several scenarios (e.g., dietary sources are oxidized at equal rates, only lipids are oxidized, etc.). With a diet in isotopic equilibrium with endogenous energy stores, a variety of birds and mammals exhibited $\delta^{13}C_{diet-breath}$ discrimination factors from −1.3‰ to +2.9‰ (Voigt et al. 2008a); the largest enrichment was in cattle (*Bos taurus*), likely due to methanogenic gut symbionts (Passey et al. 2005). Because brown bears are monograstic we assumed a discrimination factor of 0 between oxidized substrate and exhaled CO_2, similar to previous studies (Voigt et al. 2010). Based on measured $\delta^{13}C_{breath}$ values, we identified a range of probable contributions from each source to oxidation using the program IsoSource (Phillips 2012; Phillips and Gregg 2003). Contributions from each source were iteratively adjusted by 0.01 and if predicted $\delta^{13}C_{breath}$ was within 0.1‰ of measured $\delta^{13}C_{breath}$ the contributions were considered feasible (Phillips and Gregg 2003).

Results

On day 0 of the experiment, prior to the diet switch, RER values (bear A = 0.80, B = 0.77, C = 0.84, D = 0.79) indicated that bears were likely oxidizing a variable mixture of carbohydrate, lipid, and protein. Bears were switched to the experimental diet on day 0 and feeding was discontinued on day 42; however, bear A voluntarily began fasting on day 34. On day 70, bear C had an RER of 0.69, indicating lipid oxidation. Unfortunately, issues with sample collection prevented RER measurements on the remaining bears.

Values of $\delta^{13}C_{breath}$ differed among sampling occasions ($\chi^2_5 = 16.286$, $P = 0.006$). Pairwise comparisons indicated that values of $\delta^{13}C_{breath}$ changed after the diet switch, but not after the onset of fasting (Fig. 2). Values of $\delta^{13}C_{plasma}$ also differed among sampling occasions, but did not significantly change immediately after the diet switch ($F_{1,4} = 5.477$, $P = 0.010$; Fig. 2). In comparison to day 0, changes in values of $\delta^{13}C_{plasma}$ were of smaller magnitude than those in $\delta^{13}C_{breath}$ on day 28 ($t_3 = -4.166$, $P = 0.025$) and day 42 ($t_3 = -14.730$, $P < 0.001$).

Based on the calculated $\delta^{13}C_{salmon\ lipid}$ value of −29.32‰ and measured values of $\delta^{13}C_{salmon}$ and $\delta^{13}C_{oil}$ (Table 1) the predicted $\delta^{13}C_{breath}$ values for bears consuming the experimental diet ranged from −26.56‰ to −21.60‰, depending on the proportional oxidation of different sources in the diet (Table 2). Results from IsoSource based on $\delta^{13}C_{breath}$ values measured on days 28 and 42 when bears were consuming the experimental diet indicated that mean proportional contributions to oxidation ranged from 0.31 to 0.49 for salmon protein, 0.19 to 0.26 for salmon lipid, and 0.32 to 0.43 for oil (Table 3).

Discussion

In support of our expectations, after bears switched to the experimental diet of salmon protein, salmon lipid, and fish oil (referred to as ''oil''), values of $\delta^{13}C_{breath}$ indicated that dietary lipid was oxidized in greater proportion than its availability in the diet. Interestingly, most mixing models suggested this was a result of preferential oxidation of salmon lipid, rather than oil. Also in agreement with our predictions, $\delta^{13}C_{breath}$ values exhibited larger changes than $\delta^{13}C_{plasma}$ values on days 28 and 42 after the diet switch, likely because of carbon routing. On day 42 after the diet switch, food was removed from the bears. We expected that $\delta^{13}C_{breath}$ values would change to reflect abrupt reliance on endogenous lipids, and then remain stable until lipid stores were exhausted or bears resumed feeding. However, this prediction was not clearly supported, likely reflecting the complex processes of lipid deposition and mobilization.

An important constraint regarding interpretation of our data is that the experimental diet included 3 sources with distinct isotopic signatures (salmon protein, salmon lipid, and oil), yet $\delta^{13}C_{breath}$ analysis includes only 1 isotope. To allow unequivocal estimation of proportional contributions, the

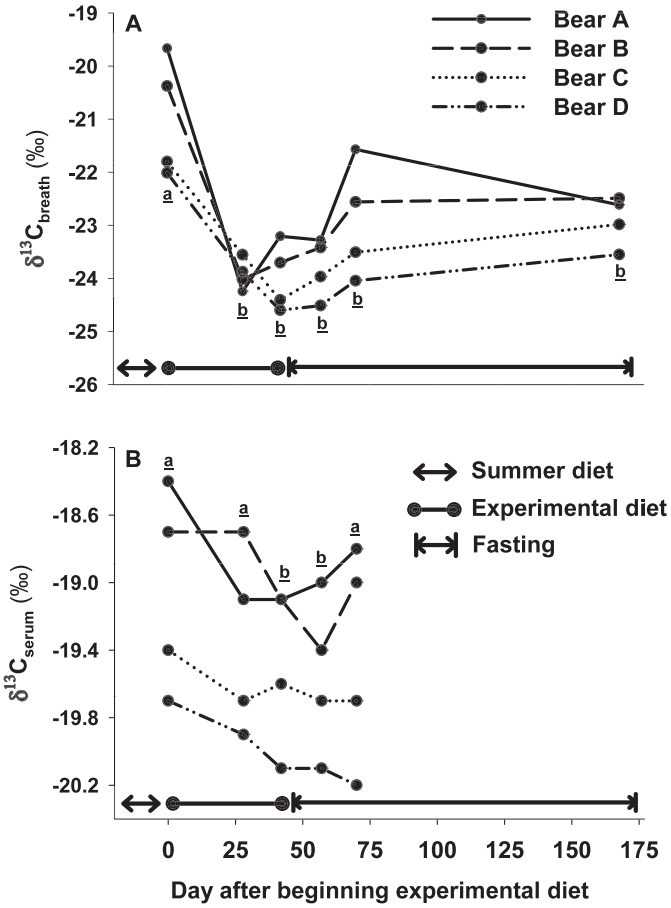

Fig. 2.—The $\delta^{13}C$ of A) exhaled carbon dioxide and B) plasma from captive brown bears that consumed an experimental diet of protein and lipid then fasted. Lowercase, underlined letters at each sampling occasion indicate significant differences among groups based on post hoc tests (A, Student–Newman–Keuls; B, Holm–Sidak). Comparisons between day 28 and 42 must be interpreted with caution because bear A voluntarily began fasting on day 34 and the other 3 bears began fasting on day 42.

TABLE 2.—Predicted $\delta^{13}C_{breath}$ values of captive brown bears, based on varying contributions of different sources in an experimental diet to oxidation. The sum of contributions for each scenario adds to 1.

Scenario	Proportional contribution of dietary sources to oxidation			Predicted $\delta^{13}C_{breath}$ (‰)
	Salmon protein	Salmon lipid	Oil	
Contributions to oxidation match proportions in diet	0.49	0.14	0.37	−23.04
No salmon protein oxidized; other materials oxidized at dietary proportions	0.00	0.28	0.72	−26.56
No salmon lipid oxidized; other materials oxidized at dietary proportions	0.57	0.00	0.43	−22.01
No oil oxidized; other materials oxidized at dietary proportions	0.78	0.22	0.00	−21.60

number of sources cannot exceed the number of analyzed isotopes by more than 1 (Phillips 2012; Phillips and Gregg 2003). However, consideration of alternative scenarios in which the contribution of each source was held to 0.00, together with estimation of feasible solutions using IsoSource, provided a reasonable assessment of the range of results.

On a dry matter basis, salmon protein constituted 0.49 of the diet and its mean predicted contribution to total oxidation ranged from 0.31 to 0.49, suggesting it was oxidized at a rate commensurate with its availability or slightly lower. In contrast, salmon lipid constituted 0.14 of the diet and its predicted contribution to oxidation ranged from 0.19–0.26, suggesting this source was preferentially oxidized. Finally, the proportion of oil in the diet was 0.37 and its predicted contribution to oxidation included 0.32–0.43, indicating that use for oxidation was not different than availability in the diet. The salmon lipid contained lower concentrations of essential FAs (linoleic acid and α-linolenic acid) than the oil (K. D. Rode, pers. obs.), and we speculate this led to preferential oxidation of salmon lipid; indeed, some studies show nonessential FAs are oxidized at a higher rate than essential FAs (Jones et al. 2008). However, other studies describe the opposite pattern, and essential FAs can be converted to nonessential FAs (Cunnane 2003), leaving the routing of nonessential FAs to oxidation an open question.

The preferential oxidation of dietary lipids over dietary protein was likely because of the greater energy density of lipids and the need to use amino acids for tissue synthesis (Brody 1999). Routing of dietary protein to synthesis may explain why $\delta^{13}C_{breath}$ showed larger changes than $\delta^{13}C_{plasma}$ on days 28 and 42 after switching to the experimental diet. Dietary isotopic values are integrated into exhaled CO_2 in less than a day (Ayliffe et al. 2004; Sponheimer et al. 2006) and into plasma proteins within approximately 10 days in black bears (Hilderbrand et al. 1996); therefore, by days 28 and 42, dietary carbon should have been incorporated into plasma as

TABLE 3.—Predicted contribution to oxidation of sources from an experimental diet fed to captive brown bears, based on $\delta^{13}C_{breath}$. IsoSource results include a scenario of the mean contribution from each source and scenarios with the lowest and highest contributions from salmon protein. The sum of contributions for each scenario adds to 1.

Bear	Days on diet	$\delta^{13}C_{breath}$ (‰)	Scenario based on IsoSource results	Proportional contribution of dietary sources to oxidation		
				Salmon protein	Salmon lipid	Oil
A	28	−24.25	$\bar{X}$ (*SD*)	0.36 (0.09)	0.24 (0.15)	0.40 (0.24)
			Lowest protein	0.19	0.00	0.81
			Highest protein	0.52	0.48	0.00
B	28	−24.02	$\bar{X}$ (*SD*)	0.39 (0.09)	0.23 (0.14)	0.38 (0.22)
			Lowest protein	0.23	0.00	0.77
			Highest protein	0.54	0.46	0.00
C	28	−23.56	$\bar{X}$ (*SD*)	0.45 (0.08)	0.21 (0.12)	0.34 (0.20)
			Lowest protein	0.31	0.00	0.69
			Highest protein	0.58	0.42	0.00
D	28	−23.88	$\bar{X}$ (*SD*)	0.40 (0.11)	0.23 (0.14)	0.37 (0.23)
			Lowest protein	0.11	0.00	0.89
			Highest protein	0.64	0.36	0.00
A	42	−23.21	$\bar{X}$ (*SD*)	0.49 (0.07)	0.19 (0.11)	0.32 (0.18)
			Lowest protein	0.36	0.00	0.64
			Highest protein	0.62	0.38	0.00
B	42	−23.70	$\bar{X}$ (*SD*)	0.43 (0.08)	0.21 (0.13)	0.36 (0.21)
			Lowest protein	0.28	0.00	0.72
			Highest protein	0.57	0.43	0.00
C	42	−24.41	$\bar{X}$ (*SD*)	0.34 (0.09)	0.25 (0.15)	0.41 (0.24)
			Lowest protein	0.17	0.00	0.83
			Highest protein	0.50	0.50	0.00
D	42	−24.60	$\bar{X}$ (*SD*)	0.31 (0.10)	0.26 (0.16)	0.43 (0.25)
			Lowest protein	0.14	0.00	0.86
			Highest protein	0.48	0.52	0.00

well as breath. The larger shift in $\delta^{13}C_{breath}$ values probably reflects the fact that carbon in exhaled CO_2 was derived primarily from dietary lipids, whereas plasma carbon mainly came from dietary protein. In fact, $\delta^{13}C_{plasma}$ values were similar to that of salmon protein, although they were slightly more enriched.

All IsoSource scenarios suggested that although dietary lipid was preferentially oxidized, a substantial proportion of dietary protein was oxidized as well (Table 3). This suggests that carnivores oxidize proteins even when lipids are readily available. Indeed, hypercarnivores—animals that naturally consume almost exclusively vertebrate prey—may not reduce the oxidation of dietary protein despite sufficient energy available in other nutrients (Rogers and Morris 2002; Zoran 2002). Protein oxidation also can be substantial in animals that primarily consume carbohydrates (McCue et al. 2010), highlighting the importance of dietary amino acids not only as gluconeogenic precursors (Eisert 2011) but as oxidative nutrients (Fig. 1).

Diet items must be digested and assimilated prior to oxidation, processes that might alter dietary influence on $\delta^{13}C_{breath}$ values. We did not account for potential differences in efficiencies of digestion and assimilation of salmon protein, salmon lipid, and oil in the experimental diet because these processes tend to be efficient in bears (Clauss et al. 2010; Pritchard and Robbins 1990). However, digestion and assimilation can differ among nutrients and monomers of nutrients (e.g., FAs—Apgar et al. 1987) and should be considered when making dietary inferences based on $\delta^{13}C_{breath}$ values.

Food was removed 42 days after beginning the experimental diet, and contrary to our expectations, $\delta^{13}C_{breath}$ values did not significantly change thereafter. Unfortunately, because bear A voluntarily began fasting on day 34 this lack of statistical difference must be interpreted with caution. In contrast, with RER values near 0.80 on day 0 of the experiment, by day 70 bear B had an RER value of 0.69, indicative of near-exclusive reliance on lipid oxidation, as expected for fasting bears. RER values can be influenced by conversion of carbohydrates to lipid (Brody 1999); however, it was beyond the scope of this study to address lipogenesis at day 0 and after that point, bears were not consuming carbohydrates. Ketogenesis also can influence RER values (Schutz and Ravussin 1980). However, levels of ketone bodies in hibernating black bears are low compared to other fasting mammals (Ahlquist et al. 1984) and efficient oxidation of ketone bodies should lead to an RER similar to the value expected for oxidation of the nutrients used for ketogenesis.

Small sample sizes may have limited our ability to detect differences in $\delta^{13}C_{breath}$ values among sampling occasions. In particular, we note that $\delta^{13}C_{breath}$ values were nearly identical between day 42 (the onset of fasting) and day 57, whereas values on days 70 and 168 appeared elevated, although the difference was not significant (Fig. 2). We cannot be certain why $\delta^{13}C_{breath}$ values did not change with fasting, but here we propose 2 plausible mechanisms based on oxidation of endogenous nutrients.

First, bears may have oxidized their own protein at a high rate, in particular between days 42 and 57, influencing $\delta^{13}C_{breath}$ values. Between summer and winter, black bears and brown bears experience an 8–20% decline in protein concentration in skeletal muscle (Hershey et al. 2008; Lohuis et al. 2007). This loss may occur during a transitional period, lasting perhaps weeks, in which fasting has begun but physiological pathways for protein conservation are not yet fully active. However, even without perfect routing of dietary amino acids to endogenous protein, the $\delta^{13}C_{endogenous\ protein}$ values of bears in our study would have been similar to the $\delta^{13}C_{salmon\ LE}$ value (−19.4‰). Values of $\delta^{13}C_{breath}$ were substantially lower (−24.60‰ to −23.21‰), indicating it is unlikely that endogenous protein was oxidized at a high rate during fasting.

Alternatively, endogenous lipids may have become the main oxidative nutrient during fasting, yet $\delta^{13}C_{breath}$ values remained stable, particularly between days 42 and 57, because recently ingested FAs were preferentially mobilized. Bears consumed an experimental diet high in unsaturated FAs for 6 weeks. These FAs were likely oxidized at a higher rate than expected (Table 3), exerting a strong influence on $\delta^{13}C_{breath}$ values, while simultaneously being deposited in endogenous lipid stores.

We speculate that after the onset of fasting on day 42, unsaturated FAs from the experimental diet were preferentially mobilized, causing $\delta^{13}C_{breath}$ values to remain unchanged. Preferential mobilization of shorter, more unsaturated FAs over longer, saturated FAs has been observed in carnivores adapted to seasonal periods of food deprivation (raccoon dog [*Nyctereutes procyonoides*—Mustonen et al. 2007]), in those not adapted (American mink [*Neovison vison*—Nieminen et al. 2006]), and in other taxa (Mongolian pheasant [*Phasianus colchicus mongolicus*—Mustonen et al. 2009]). Triacylglycerols containing short, unsaturated FAs are more polar and thus more likely to be positioned at the periphery of the intracellular lipid droplet because of interaction with the polar, aqueous cytosol. In that configuration, these triacylglycerols would be more accessible to lipases, which mobilize FAs (Raclot 2003; Raclot and Groscolas 1993).

In our study, after bears began fasting, the FAs accumulated from the experimental diet would eventually be depleted. Subsequently, FAs deposited from the carbohydrate-rich summer diet would contribute an increasing proportion of the oxidative nutrients, and $\delta^{13}C_{breath}$ values would slowly change to reflect this shift. Our data do not support a significant change in $\delta^{13}C_{breath}$ values after extended fasting (Fig. 2), but we suspect larger sample sizes and more frequent sampling may be required to identify such a trend.

In conclusion, our results demonstrate that $\delta^{13}C_{breath}$ values provide a sensitive indicator of diet switching in a captive carnivore, supporting the application of these techniques to free-ranging animals. We also conclude that even when energy-rich lipids are abundant in the diet, ingested proteins are oxidized at a substantial rate. Because $\delta^{13}C_{breath}$ values can be influenced by diet selection, diet nutrient composition, and

fasting state, interpretation of $\delta^{13}C_{breath}$ values should be complemented with additional data where possible. This may include behavioral observation, nutrient analysis of the diet, assessment of body condition and reproductive state, and measurement of RER. For example, Hobson et al. (2009) suggested that polar bears in western Hudson Bay gained little energetic benefit from consuming terrestrial food sources, such as berries, on the basis of $\delta^{13}C_{breath}$ values. Their inference that bears primarily oxidize endogenous lipid despite consuming carbohydrate-rich vegetation concurs with previous research (Hobson and Stirling 1997; Ramsay and Hobson 1991; reviewed in Rode et al. 2010), but would have been strengthened by RER measurements of approximately 0.70.

Inferences of fasting state based on $\delta^{13}C_{breath}$ values are more complex than those of diet; contrary to our expectations, values of $\delta^{13}C_{breath}$ did not respond to food deprivation. This highlights the importance of lipid metabolism in fasting animals, and suggests that selective mobilization of stored FAs may influence $\delta^{13}C_{breath}$ values. Thus, future studies should consider documenting potential changes in FA profiles of adipose tissue during fasting, and measuring FA-specific isotopic signatures.

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