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A Mass Balance Approach to Identify and Compare Differential Routing of ^{13}C-Labeled Carbohydrates, Lipids, and Proteins In Vivo

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ABSTRACT

All animals route assimilated nutrients to their tissues where they are used to support growth or are oxidized for energy. These nutrients are probably not allocated homogeneously among the various tissue and are more likely to be preferentially routed toward some tissues and away from others. Here we introduce an approach that allows researchers to identify and compare nutrient routing among different organs and tissues. We tested this approach by examining nutrient routing in birds. House sparrows *Passer domesticus* were fed a meal supplemented with one of seven ^{13}C-labeled metabolic tracers representing three major classes of macronutrients, namely, carbohydrates, amino acids, and fatty acids. While these birds became postabsorptive (2 h after feeding), we quantified the isotopic enrichment of the lean and lipid fractions of several organs and tissues. We then compared the actual ^{13}C enrichment of various tissue fractions with the predictions of our model to identify instances where nutrients were differentially routed and found that different classes of macronutrients are uniquely routed throughout the body. Recently ingested amino acids were preferentially routed to the lean fraction of the liver, whereas exogenous carbohydrates were routed to the brain and the lipid fraction of the liver. Fatty acids were definitively routed to the heart and the liver, although high levels of palmitic acid were also recovered in the adipose tissue. Tracers belonging to the same class of molecules were not always routed identically, illustrating how this technique is also suited to examine differences in nonoxidative fates of closely related molecules. Overall, this general approach allows researchers to test heretofore unexamined predictions about how animals allocate the nutrients they ingest.

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Introduction

Assimilated nutrients, such as carbohydrates, lipids, and amino acids, undergo one of three general fates: (1) they can be used as fuel to meet an animal's immediate energy requirements; (2) they can be incorporated into tissues, where they serve structural or energy storage functions to be oxidized later; or (3) they can bypass catabolic processes altogether and be routed to sites where their use is irreversible, for example, in conspicuous fur and feathers or in less conspicuous oily secretions and sloughed skin cells. Assignation of nutrients to different tissues and fates is thought to be proximately influenced by an animal's nutritional, developmental, and reproductive status and ultimately by its life history (Martin 1987; Dunham et al. 1989; Ricklefs and Wikelski 2002).

For nearly 2 decades researchers have measured the $^{13}CO_2$ enrichment in exhaled breath of animals fed a diet enriched in ^{13}C to determine the rates at which particular exogenous nutrients are oxidized. These studies typically provided semiquantitative information about the oxidation of one nutrient, but occasionally two closely related molecules were compared. Building on this background, we recently formalized an approach that allows quantification of the oxidative kinetics of a wide range of ^{13}C nutrients in virtually any air-breathing animal (McCue et al. 2010*a*) and have since shown that several factors influence the oxidative fates of exogenous nutrients (McCue 2011). Here we outline an approach that uses the mass balance of stable isotope–labeled nutrients to determine their nonoxidative fate and hence how specific nutrients are preferentially

routed in living animals. We developed a null model that predicts nutrient routing, assuming that unoxidized tracer molecules are homogeneously distributed among the exchangeable pool of atoms in the body (Pauli et al. 2009; McCue 2011). Measured deviations from these predictions indicate nutrient routing toward (positive routing) a particular tissue, thereby allowing researchers to (1) identify the specific organs, tissues, or tissue fractions to which specific nutrients are preferentially routed and (2) compare the nutrient-routing vectors among different tissues at different points in time. We use this approach to characterize the nutrient routing of various amino acids, fatty acids, and carbohydrates among the tissues of an omnivorous bird, the house sparrow *Passer domesticus*.

Methods

Animals

In 2008 and 2009, 32 house sparrows *Passer domesticus* L. were captured with mist nets at Midreshet Ben-Gurion, Israel (30°51′8.27″N, 34°47′0.24″E). The birds were banded with uniquely numbered leg bands and quarantined in a large, permanent outdoor aviary (4 m × 3 m × 2 m [L × W × H]), where they were fed a diet of mixed millet seeds (approximately 12% protein and 5% lipid as dry mass; Williams and Ternan 1999) and provided with tap water, both ad lib., for a minimum of 45 d. Crushed chicken eggshells, vitamin-supplemented water, and fresh lettuce were also provided once a week. Males and females were housed together, but reproductive activity was not observed.

At least 1 mo before experiments, the birds were administered two deworming treatments, 1 wk apart, to eliminate intestinal parasites that might influence oxidative dynamics of tracers. Birds were orally gavaged with a dose of Ivermectin (220 μg kg^{-1} in 0.5 mL water), using a 15-g silicon-tipped polyethylene feeding tube (FTP-15-78; Instech Solomon, Plymouth Meeting, PA) attached to a 1.0-mL syringe. This was followed a week later by a dose of Fenbendazole (30 mg kg^{-1} in 0.5 mL water). After deworming, the sparrows were transferred to neighboring outdoor aviaries (1.5 m × 1.5 m × 2.5 m), with eight to 12 individuals in each.

Nutrient Tracers and Animal Tissues

Sparrows with full crops were selected from the aviary at between 1000 and 1400 hours and were orally gavaged with 20 mg of one of seven isotopically labeled molecules (i.e., 1-^{13}C D-glucose, 98–99%; 1-^{13}C D-fructose, 99%; 1-^{13}C L-leucine, 99%; 1-^{13}C glycine, 99%; 1-^{13}C palmitic acid, 99%; 1-^{13}C stearic acid, 99%; 1-^{13}C oleic acid, 99%; Cambridge Isotope Laboratories, Andover, MA) suspended in 200 μL of sunflower seed oil. A control treatment consisted of 200 μL of sunflower seed oil alone. Treatments were administered in a random order, and four birds were used for each treatment group. Administering the labeled nutrients directly into the gastrointestinal tract by gavage ensured that the tracer kinetics included key natural metabolic processes such as intestinal absorption, circulatory transport, perhaps liver metabolism, and finally incorporation into tissue (McCue 2011).

Two hours after isotope administration, birds were killed by decapitation, and trunk blood was collected. Tissues including heart, liver, pectoral muscle, brain, intestine, and adipose tissue were harvested within 5 min of death. All tissues were frozen and then lyophilized for 72 h. Dried tissues were homogenized using a mortar and pestle and stored at −20°C. Whole heart, liver, pectoral muscle, and brain were used to assess routing of the three fatty acid tracers. To assess the routing of the two carbohydrate and two amino acid tracers, we examined the isotopic enrichment of lean and lipid fractions of the liver and brain, as well as lean fractions of the pectoral muscle and heart. Isotope analyses were done on whole blood and adipose tissue.

The separation of lipid fractions from lipid-rich tissue samples is recommended for standardizing comparisons at natural abundances of ^{13}C (Petelle et al. 1979; Kelly 2000; Harvey et al. 2002; Post et al. 2007; Vollaire et al. 2007; Logan et al. 2008); however, this step is less critical for artificial enrichments (McCue 2011). Nevertheless, we did separate some tissues into lean and lipid fractions to identify postabsorptive modification of the tracers (e.g., through gluconeogenesis, lipogenesis, etc.). We placed approximately 100 mg of whole-tissue homogenate into Teflon-coated vials containing 8 mL of a 2 : 1 solution of chloroform : methanol, heated to 60°–70°C, and agitated samples for 1 h with a magnetic stirrer. Vials were then centrifuged at 3,000 rpm for 5 min, and the lipid-containing supernatant was removed. Tissue pellets were dried in a convection oven at 60°C for 24–36 h, and the lipid fraction from each liver and brain was recovered by drying the supernatant to constant mass under a stream of N_2 (Table 1). Homogenized tissue samples and lipid fractions were weighed to ± 0.0001 g and loaded into tin cups for isotope analysis.

Carbon isotope analysis was done at the U.S. Environmental Protection Agency Atlantic Ecology Division laboratory, using a Carlo-Erba NA 1500 series II elemental analyzer interfaced with an Elementar Optima isotope ratio mass spectrometer. Samples were burned at 1,020°C in the presence of a chromic oxide catalyst. Evolved CO_2 was sent to the mass spectrometer

Table 1: Mean isotope signatures (± 1 SD) of house sparrow tissues from control birds ($n = 4$) orally gavaged with 200 μL of sunflower seed oil alone

	δ^{13}C Signature in Tissue (fraction)		
Tissue	Whole Tissue	Lean	Lipid
Adipose	−17.20 (.07)	NA	NA
Blood	−12.24 (.07)	NA	NA
Brain	−13.60 (.85)	−11.17 (.41)	−16.53 (.61)
Heart	−12.80 (.58)	−11.26 (.27)	NA
Intestine	−11.36 (.12)	−12.03 (.31)	NA
Liver	−14.42 (.47)	−11.92 (.12)	−18.03 (.03)
Pectoral	−12.15 (.30)	−11.14 (.38)	NA

Note. Some tissues were further separated into lean and lipid fractions in a heated 2 : 1 solution of chloroform : methanol. NA, not available.

for the measurement of carbon isotope ratios. We used two internal laboratory standards for every 10 unknown samples in a sequence. Stable-carbon isotope ratios are expressed in delta notation as parts per thousand (‰) and compared with Vienna Pee Dee Belemnite (Craig 1957). The internal standard used has a long-term average $\delta^{13}C$ measurement precision of ± 0.17‰. Combined with an internal evaluation of the reproducibility of tissue and isotope tracer $\delta^{13}C$ measurements, $\delta^{13}C$ measurements in this study are accurate to ± 0.40‰.

Calculating Nutrient Routing

Patterns of nutrient routing were determined by comparing measured $\delta^{13}C$ values after dosing (i.e., $\delta^{13}C_{postdose}$) with expected $\delta^{13}C$ (i.e., $\delta^{13}C_{null\ model}$), assuming that tracers were homogeneously distributed among tissues. Each tissue had a unique $\delta^{13}C$ value, so we added the estimated increase in $\delta^{13}C$ (i.e., $\delta^{13}C_{enrichment}$) to the measured background $\delta^{13}C$ values (i.e., $\delta^{13}C_{control}$) for each tissue to calculate $\delta^{13}C_{null\ model}$.

Since we used artificially enriched tracers, it is proper to calculate the expected incremental increase in ^{13}C (i.e., $AP^{13}C_{enrichment}$) in atom percent (Slater et al. 2001):

$$AP^{13}C_{enrichment} = \left[\frac{^{13}C_{exogenous}}{C_{exchangeable} + {}^{13}C_{exogenous}}\right] \cdot 100, \qquad (1)$$

where $C_{exchangeable}$ represents the size of the exchangeable carbon pool (see Eq. [2]) and $^{13}C_{exogenous}$ represents the amount of ^{13}C added to the exchangeable carbon pool (see Eq. [3]). Because $AP^{13}C_{enrichment}$ was so small, we converted $AP^{13}C_{enrichment}$ into delta notation to calculate $\delta^{13}C_{enrichment}$ (Slater et al. 2001).

The size of the exchangeable carbon atom pool (i.e., $C_{exchangeable}$) was defined as

$$C_{exchangeable} = (m_{live} - m_{water} - m_{feather}) \cdot F_{body\,C}, \qquad (2)$$

where m_{live}, m_{water}, and $m_{feather}$ are the live mass (immediately after dosing), water mass, and feather mass of the animal in milligrams, respectively. Water mass was assumed to account for 53% of m_{live} (Mahoney and Jehl 1984; Yokota et al. 1992; Karasov and Pinshow 1998; Amat et al. 2007), and feathers were assumed to account for 7% of m_{live} (Karasov and Pinshow 1998; Koutsos et al. 2001); $F_{body\,C}$ is the mass fraction of carbon in the dried body and was assumed to be 0.45 (a value typical for temperate and tropical vertebrates; T. Millican, personal communication).

The amount of administered, assimilated, and retained ^{13}C atoms (i.e., $^{13}C_{exogenous}$) was defined as

$$^{13}C_{exogenous} = m_{dose} \cdot F_{^{13}Cdose} \cdot F_{assimilated} \cdot (1 - F_{oxidized}), \qquad (3)$$

where m_{dose} is the mass of the tracer dose in milligrams; $F_{^{13}Cdose}$ is the mass fraction of ^{13}C in each tracer molecule; $F_{assimilated}$ is the fraction of the assimilated tracer dose, estimated to be 0.90 (Renner and Hill 1961; Hurwitz et al. 1973; Chung and Baker 1992; Bairlein and Simons 1995; Caviedes-Vidal and Karasov 1996; Chediack et al. 2006); and $F_{oxidized}$ is the fraction of the tracer dose that was oxidized, as determined by breath testing (glycine, 0.452; leucine, 0.128; glucose, 0.059; fructose,

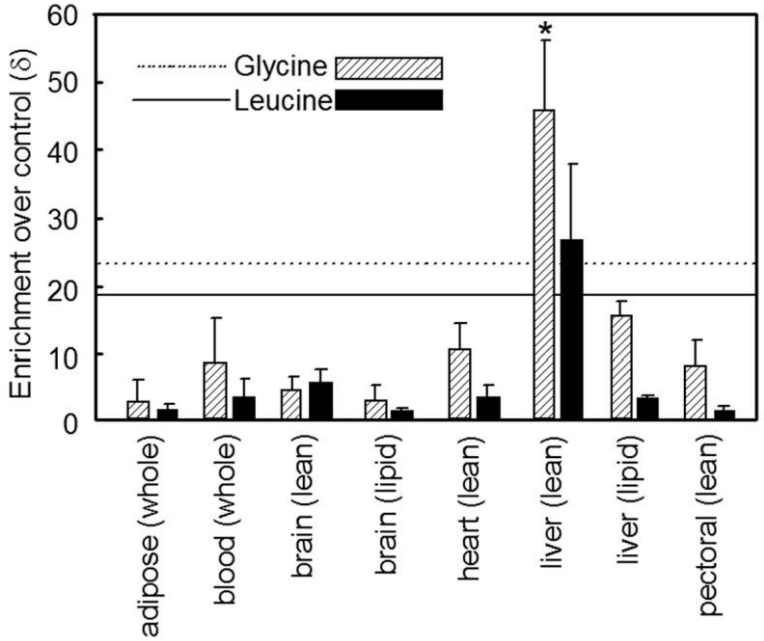

Figure 1. Enrichment of ^{13}C in the tissues of house sparrows fed amino acid tracers. The dashed and solid lines represent the calculated enrichment, namely, 23 and 19‰ for glycine and leucine, respectively, assuming that the tracers were homogeneously distributed among all tissues. Asterisks indicate values that are statistically above these lines and evidence for preferential routing of a nutrient to that tissue. Error bars indicate 1 SD.

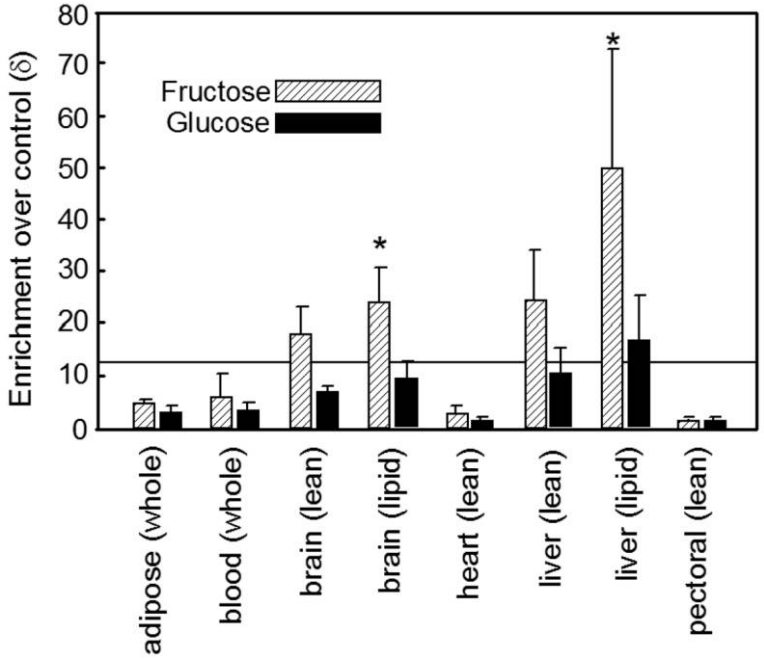

Figure 2. Enrichment of ^{13}C in the tissues of house sparrows fed carbohydrate tracers. The solid line represents the calculated enrichment, that is, 12‰ for both, assuming tracers were homogeneously distributed among all tissues. Asterisks indicate values that are statistically above these lines and evidence for preferential routing of a nutrient to that tissue. Error bars indicate 1 SD.

0.059; palmitic acid, 0.007; oleic acid, 0.006; and stearic acid, 0.002; McCue et al. 2010*b*).

Measured values were compared with the null model for each tracer by using one-sample *t*-tests with a critical $\alpha = 0.05$. When $\delta^{13}C_{postdose}$ was significantly higher than $\delta^{13}C_{null\ model}$ for a tissue sample, we concluded that nutrients were routed preferentially to that tissue.

Results

The $\delta^{13}C_{control}$ (i.e., background values) of whole tissues ranged from −17.20‰ in adipose tissue to −11.36‰ in intestine (Table 1). Lean-tissue fractions of lipid-rich tissue, such as brain and liver, averaged 2.47‰ more enriched than whole tissues, but the lean fractions of both striated and smooth muscle tissues (e.g., heart, pectoral muscle, intestine) differed by an average of only 0.63‰ from whole tissues. The lipid fractions of brain and liver averaged 3.27‰ more depleted than the respective whole organs and resembled values for whole adipose tissue.

By 2 h after administration, $\delta^{13}C_{postdose}$ ranged from −0.25‰ in whole brain tissue of birds dosed with stearic acid to 49.90‰ in the lipid fraction of the livers of birds dosed with ^{13}C-fructose. On average, the tissues of birds dosed with tracers had a $\delta^{13}C$ of 13.22‰, a value >25‰ higher than the $\delta^{13}C_{control}$, suggesting that the isotopic enrichments that we used were sufficient to be used as isotopic markers (Pauli et al. 2009).

The $\delta^{13}C_{enrichment}$ ranged from 3 to 23‰ for stearic acid and glycine, respectively. The incremental $\delta^{13}C_{enrichment}$ was added to the $\delta^{13}C_{control}$ values to establish the $\delta^{13}C_{null\ model}$, assuming homogeneous routing. Isotopic enrichments above background values were detected in all tissues after label administration. The $\delta^{13}C_{null\ model}$ was an effective indicator of preferential nutrient routing; on average, about one-third of the tissues had mean values above the $\delta^{13}C_{null\ model}$. Similar to other breath-testing studies examining the oxidative fates of nutrients (McCue et al. 2010*b*, 2011), our study found general similarities in the nonoxidative fates of molecules belonging to the same class. For example, breath testing revealed that amino acids were oxidized far more rapidly than the other tracers, and they were found at the highest concentrations in the liver. Carbohydrates were oxidized less extensively than amino acids but more than fatty acids and were found in the highest concentrations in the liver and brain. Fatty acids were oxidized less than the other two classes of tracer molecules and were found at the highest concentrations in the heart and liver.

The ^{13}C from the glycine tracer was preferentially routed to the lean fraction of the liver ($t = 4.385$, df = 3, $P = 0.022$; Fig. 1). The $\delta^{13}C_{postdose}$ enrichment of the leucine tracer in the lipid fraction of the liver did not exceed values indicative of preferential routing; however, these values were an average of 16.6‰ above $\delta^{13}C_{control}$, suggesting the possibility that a significant amount of glycine was used for lipogenesis. The ^{13}C from amino acid tracers was found in the lowest concentrations in the brain and adipose tissue.

The ^{13}C from fructose was preferentially routed to the lipid fractions of the brain ($t = 3.585$, df = 3, $P = 0.037$) and liver

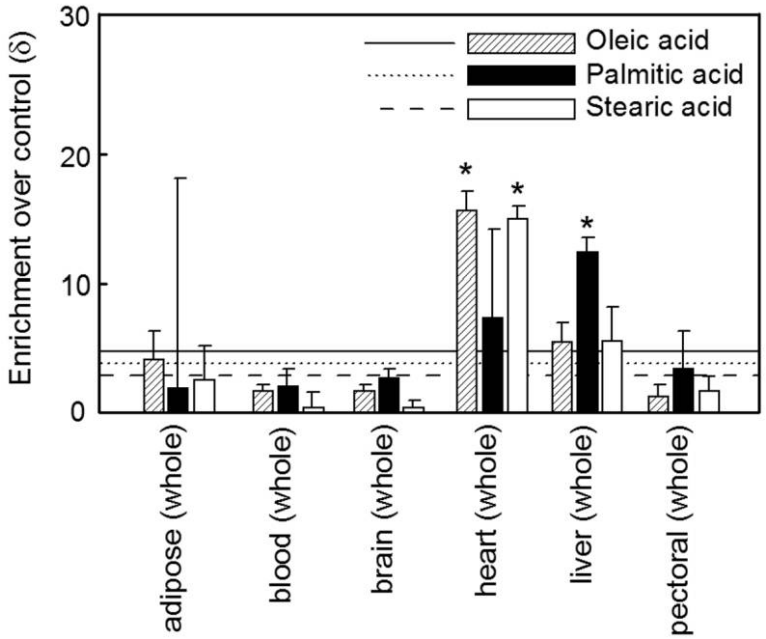

Figure 3. Enrichment of ^{13}C in the tissues of house sparrows fed fatty acid tracers. The dashed, dotted, and solid lines represent the calculated enrichment, namely, 3, 4, and 5‰ for stearic acid, palmitic acid, and oleic acid, respectively, assuming that the tracers were homogeneously distributed among all tissues. Asterisks indicate values that are statistically above these lines and evidence for preferential routing of a nutrient to that tissue. Error bars indicate 1 SD.

($t = 3.351$, df = 3, $P = 0.044$), but preferential routing of ^{13}C from glucose was not detected in any of the examined tissues (Fig. 2). Carbohydrates were found in the lowest concentrations in the cardiac and pectoral muscles.

Preferential routing of fatty acids was detected in two tissues (Fig. 3) and was dependent on the specific fatty acid examined. For example, ^{13}C from palmitic acid was preferentially routed to the liver ($t = 20.910$, df = 3, $P < 0.001$), but oleic and stearic acid were preferentially routed to the heart ($t = 14.528$, df = 3, $P < 0.001$ and $t = 41.875$, df = 3, $P < 0.001$, respectively). The highest measured $\delta^{13}C$ value following dosing with fatty acid tracers (i.e., 21.79‰) was found in the adipose tissue of an individual administered ^{13}C-palmitic acid, but because of the high interindividual variability, we were unable to confirm positive routing to that tissue. The lowest concentrations of fatty acid tracers were recovered in the blood and brain.

Discussion

In light of current knowledge about nutrient allocation in birds (Klasing 1998; McWilliams et al. 2004), we expected that amino acids would be preferentially routed to the liver and pectoral muscle and routed away from the brain and adipose tissue. Our data generally support this expectation; amino acids were positively routed to the lean fraction of liver within 2 h of ingestion, presumably because they were being incorporated into peptides and proteins. Relative to leucine, however, the comparatively high levels of glycine in the lean and lipid liver fractions suggest that this nonessential nutrient was also being extensively deaminated for oxidation and lipogenesis. Simultaneous monitoring of quantitative and qualitative differences in amino acid transporters in tissues might offer mechanistic insight into the observed allocation patterns (Humphrey et al. 2004).

We expected that monosaccharides would be preferentially routed to hepatic glycogen and adipose tissue and routed away from muscle tissue. Our results showed that fructose was positively routed to the liver and brain within 2 h of ingestion; in the lean and lipid fractions of the liver, this outcome was likely the result of glycogenesis and lipogenesis, respectively. Given that similar amounts of exogenous glucose and fructose were oxidized by sparrows (McCue et al. 2011), the fact that all of the analyzed tissue fractions had higher concentrations of ^{13}C-fructose in the treated birds was somewhat surprising. If $^{13}CO_2$ breath testing is not done in parallel with this approach, it would be useful to verify the calculated $AP^{13}C_{enrichment}$ on a subset of animals immediately after dosing. Moreover, examination of additional organs and tissues at different times after tracer administration could be useful to determine the routing patterns for this "missing" glucose. The lowest levels of ^{13}C enrichment in carbohydrate-dosed birds were found in the skeletal muscle, suggesting that the birds were not using exogenous sugars to replenish muscle glycogen stores. Because the birds in this study were well nourished before being dosed with the tracer, additional studies are required to determine whether undernourished animals route nutrients in the same way (Podlesak and McWilliams 2006, 2007; McCue et al. 2010*a*).

We expected that fatty acids would be preferentially directed toward adipose and hepatic tissue and away from muscles. We found preferential allocation of two fatty acids to the heart and another fatty acid to the liver. However, while we expected that all three of the fatty acids would be positively routed to adipose tissue, palmitic acid had the greatest propensity to be routed to adipose tissue. The high levels of palmitic acid in adipose tissue apparently make this molecule less available as a fuel for cardiac tissue (Fig. 3). Fatty acid allocation to muscles depended on muscle type. Fatty acids were routed away from skeletal muscle and preferentially routed to the heart within 2 h of ingestion. This pattern bolsters the well-documented preference for fatty acids as a metabolic fuel by cardiac tissue (Ballard et al. 1960; Eser et al. 1967; Crass et al. 1970; Evans et al. 2000).

As a starting point of our mass balance approach, outlined above, we assumed a constant across-the-board assimilation efficiency of 0.90 for all nutrients and a fractional carbon content in the dry body of 0.45. These assumptions may be appropriate for individuals feeding on well-balanced diets and in good body condition, as in this experiment; however, under less favorable conditions, both assimilation efficiency (Karasov and McWilliams 2005) and body carbon (McCue 2010) may vary. Fortunately, the same labeled tracers can be used to estimate assimilation efficiency (Afik and Karasov 1995; Caviedes-Vidal and Karasov 1996) and fractional carbon content of tissue, as long as tissues are sampled frequently over time (McClelland and Montoya 2002; Chamberlain et al. 2006; McCarthy et al. 2007).

Analysis of particular compounds in tissues (e.g., specific amino acids, fatty acids, carbohydrates) would further elucidate how ingested compounds are metabolically routed and biochemically transformed (McClelland and Montoya 2002; Chamberlain et al. 2006; McCarthy et al. 2007). Our tissue fraction–specific analyses distinguished between lean and lipid tissue fractions. We were therefore able to distinguish between ^{13}C atoms initially attached to glucose that remained in a lean-carbohydrate fraction and those that were converted into fatty acid or glycerol and were subsequently incorporated into the lipid fraction (Obeid and Emery 1997; Obeid et al. 2000; Gaye-Siessegger et al. 2004). Additional tissue separation approaches might include extraction of lean fractions with trichloroacetic acid or separation of the lipid fraction into polar and neutral components (Guglielmo et al. 2002; McCue et al. 2009; Ben-Hamo et al. 2011). Despite repeated calls in the literature, we are unaware of any attempts to combine mass balance and compound specific approaches of artificially enriched nutrients (Bos et al. 2003; Wolfe and Chinkes 2005; Conceicao et al. 2007; Martinez del Rio et al. 2009). Such compound-specific analyses could validate some of the mechanisms we propose here (e.g., gluconeogenesis and lipogenesis of amino acid substrates).

We have shown that small amounts of purified ^{13}C-labeled tracers can be used to identify the preferential routing of exogenous nutrients into tissues. By mathematically defining "preferential routing," this approach opens the door to future comparative investigations into how animals allocate the nutrients they ingest and can be used to test specific predictions about how endogenous and exogenous factors shape these decisions (McCue 2011). For example, how does nutrient routing differ between species when animals are growing normally compared with their growing after a bout of reduced food intake and during the subsequent compensatory growth? Why do trophic fractionation rates differ between species when animals are fed different diets with the same isotope values? What changes in nutrient routing precede seasonal hibernation? What changes in nutrient routing accompany ontogenetic diet shifts or metamorphosis? Which dietary nutrients are primarily used to build muscle and fat tissues, how does this differ between tissues within the same animal, and how does this routing of nutrients change with environmental conditions (e.g., food availability, warmer or colder temperatures)? These same models can be modified to test other null hypotheses other than an entirely homogeneous distribution of exogenous nutrients to tissues, such as the "scrambled egg premise" eschewed by Van Der Merwe (1982). For example, tissues differ in overall size of their carbon pools and in their turnover rates (Bauchinger and McWilliams 2010), which could affect the rate of metabolic routing. Since animals are rarely, if ever, in a physiological steady state (Steendam et al. 2004; Wolfe and Chinkes 2005; Conceicao et al. 2007; McCue 2011), future attempts to characterize exogenous nutrient routing should include temporal as well as spatial tissue–specific components.

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