

## Tracking the oxidative kinetics of carbohydrates, amino acids and fatty acids in the house sparrow using exhaled $^{13}\text{C}\text{O}_2$

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### SUMMARY

Clinicians commonly measure the  $^{13}\text{C}\text{O}_2$  in exhaled breath samples following administration of a metabolic tracer (breath testing) to diagnose certain infections and metabolic disorders. We believe that breath testing can become a powerful tool to investigate novel questions about the influence of ecological and physiological factors on the oxidative fates of exogenous nutrients. Here we examined several predictions regarding the oxidative kinetics of specific carbohydrates, amino acids and fatty acids in a dietary generalist, the house sparrow (*Passer domesticus*). After administering postprandial birds with 20 mg of one of seven  $^{13}\text{C}$ -labeled tracers, we measured rates of  $^{13}\text{C}\text{O}_2$  production every 15 min over 2 h. We found that sparrows oxidized exogenous amino acids far more rapidly than carbohydrates or fatty acids, and that different tracers belonging to the same class of physiological fuels had unique oxidative kinetics. Glycine had a mean maximum rate of oxidation ( $2021 \text{ nmol min}^{-1}$ ) that was significantly higher than that of leucine ( $351 \text{ nmol min}^{-1}$ ), supporting our prediction that nonessential amino acids are oxidized more rapidly than essential amino acids. Exogenous glucose and fructose were oxidized to a similar extent (5.9% of dose), but the time required to reach maximum rates of oxidation was longer for fructose. The maximum rates of oxidation were significantly higher when exogenous glucose was administered as an aqueous solution ( $122 \text{ nmol min}^{-1}$ ), rather than as an oil suspension ( $93 \text{ nmol min}^{-1}$ ), supporting our prediction that exogenous lipids negatively influence rates of exogenous glucose oxidation. Dietary fatty acids had the lowest maximum rates of oxidation ( $2\text{--}6 \text{ nmol min}^{-1}$ ), and differed significantly in the extent to which each was oxidized, with 0.73%, 0.63% and 0.21% of palmitic, oleic and stearic acid tracers oxidized, respectively.

Key words: catabolism, glycolysis,  $\beta$ -oxidation, birds, nutrition, physiological fuels, metabolic rates, carbon stable isotope.

### INTRODUCTION

All animals require a continual input of substrates, namely carbohydrates, lipids and proteins, to provide the structural components and energy required to carry out the basic processes of life, including growth and reproduction. By supplementing an animal's diet with known amounts of  $^{13}\text{C}$ -labeled molecules one can investigate the time course over which different exogenous physiological fuels are oxidized to meet an animal's energy demands – a procedure known as 'breath testing'. Breath testing, using orally or intravenously administered stable isotope tracers, has been regularly used for two decades to diagnose infections, malabsorption and metabolic disorders in humans (Amarri and Weaver, 1995; Bodamer and Halliday, 2001; Wetzal, 2005), but the growing number of commercially available tracer molecules (Amarri and Weaver, 1995; Bodamer and Halliday, 2001; Ishihara et al., 2002) and the reduced costs and high throughput of continuous-flow isotope-ratio mass spectrometers (Martinez del Rio et al., 2009; McCue, 2008) makes breath testing increasingly available to comparative physiologists (Hatch et al., 2002a; McCue and Pollock, 2008).

Recently, the number of studies employing breath testing to address questions about species other than laboratory rats and mice and humans, has increased substantially. Most of these studies used experimental approaches where animals were switched between diets derived from  $\text{C}_3$  or  $\text{C}_4$  plants (Ayliffe et al., 2004; Hatch et al., 2002b; Passey et al., 2005; Sponheimer et al., 2006; Starck et

al., 2004; Voigt et al., 2008a; Welch et al., 2006). Although the approach of using diets that differ in natural abundances of  $^{13}\text{C}$  is inexpensive, it faces two potential limitations, namely accuracy and specificity. The accuracy of continuous-flow isotope-ratio mass spectrometers can be as high as  $\pm 0.1\text{--}0.2\%$  (Buchmann et al., 1998; Matthews and Hayes, 1978; Schoeller et al., 1980), but in bench-top analyzers designed for clinical settings (Wetzal, 2005), it can be as low as  $\pm 1.0\%$  or more, although the reported precision is often less (Sponheimer et al., 2006; Voigt et al., 2008a). In studies where the isotopic difference between two diets may be 8–12%, such analytical errors may obscure small differences in rates of substrate oxidation.

Also, although naturally enriched, experimental diets may show statistically significant differences in  $^{13}\text{C}$  signatures, without compound-specific isotope analyses it is impossible to characterize how  $^{13}\text{C}$  is distributed among the various classes of macromolecules (e.g. carbohydrates, lipids and proteins) (Podlesak and McWilliams, 2006; Podlesak and McWilliams, 2007). Furthermore without using NMR, one cannot ascertain how isotopes of interest are distributed throughout molecules. As a result, two experimental diets with identical isotopic signatures could have very different macronutrient composition. These and other limitations to using naturally enriched diets have been discussed in the literature (Amarri and Weaver, 1995; Hatch et al., 2002a; Martinez del Rio et al., 2009; Podlesak and McWilliams, 2006; Sanchez-Guzman et al., 2004; Voigt et al., 2008b).

In recent studies on nectarivorous bats and birds, researchers have overcome the obstacle of specificity by feeding the animal a single organic molecule, usually a solution of a single monosaccharide or disaccharide derived from C<sub>3</sub> or C<sub>4</sub> plants (Carleton et al., 2006; Voigt et al., 2008a; Voigt and Speakman, 2007; Welch et al., 2006; Welch et al., 2008; Welch and Suarez, 2007). However, given the comparatively small differences in isotopic signatures of such diets, such an approach may still lack the precision required to identify minor, but important differences in oxidative kinetics. Moreover, broad comparisons about how different physiological fuels are oxidized are not feasible given that most vertebrates do not ingest nectar meals, but rather consume diets containing a mixture of carbohydrates, proteins and lipids, the proportions of which can vary considerably (Karasov and Diamond, 1988).

Most studies in which the oxidative fates of exogenous nutrients have been characterized have employed mammals, including humans, mice, rats and pigs, but little is known of their fates in other taxa, particularly birds. Consequently, we used breath testing to test our general hypotheses that different exogenous metabolic fuels are oxidized at unique rates in house sparrows (*Passer domesticus*), a species known to be a dietary generalist (Anderson, 2006; Lendvai et al., 2004). Specifically, we gavaged postprandial sparrows with one of seven <sup>13</sup>C-labeled tracers and tested the following predictions.

(1) Carbohydrates. Studies of exercising humans have shown that some exogenous carbohydrates are oxidized at lower rates than others. For example, Adopo et al. (Adopo et al., 1994) found that 14% less fructose than glucose was oxidized by individuals fed a solution containing a 1:1 ratio of glucose to fructose. Massicotte et al. (Massicotte et al., 1986) found that 25% more exogenous glucose than fructose was oxidized over a 3-h period after individuals were fed either glucose or fructose tracers. Given that glucose and fructose are both assimilated by birds with high efficiency (Bismut et al., 1993; Ferraris, 2001; Jackson et al., 1998; Martinez del Rio et al., 1989), but that fructose can bypass phosphofructokinase, a limiting step of glycolysis (Bismut et al., 1993; Yeh and Leveille, 1971), we tested the prediction that maximum instantaneous and cumulative rates of exogenous oxidation are higher for fructose than glucose in sparrows.

Because many purified amino and fatty acid (FAs) tracers, have low solubility in water, they are often suspended in an oil medium prior to gavage (Wetzel, 2005). To be consistent, all of the tracers used in this study were administered as an oil suspension; however it is possible that the oil could influence the rates at which some water-soluble tracers, such as glucose, are oxidized. For example, Elia et al. (Elia et al., 1992) and Schrauwen et al. (Schrauwen et al., 2000) found that rates of exogenous glucose tracer oxidation decreased when humans consumed a high-fat diet. Moreover, Romijn et al. (Romijn et al., 1995) found that in exercising humans given infusions of palmitate, rates of exogenous glucose oxidation were reduced, which led to a concomitant increase in FA oxidation. These effects on the oxidative kinetics of carbohydrates are thought to result from direct competitive interactions between oxidation of FAs and carbohydrates (Ferrannini et al., 1983), as well as the indirect effects of FA availability on circulating insulin levels (Sidossis and Wolfe, 1996). Therefore, we examined whether the gavage medium influences the oxidation rate of exogenous glucose, and predicted that rate of oxidation is higher when administered in an aqueous solution than when suspended in oil.

(2) Amino acids. Birds, like most animals, are not known to store protein in specialized organs and tissues for extended periods (Jenni and Jenni-Eiermann, 1998); this creates a situation where exogenous

amino acids that are absorbed in excess of the body's immediate requirements become quickly transaminated or deaminated and oxidized (Brown and Cameron, 1991; Cortiella et al., 1988; Coulson et al., 1978; Coulson and Hernandez, 1968; McCue et al., 2005). Moreover, because mixed protein diets contain both essential and nonessential amino acids, it has been suggested that ingested nonessential amino acids are preferentially oxidized over essential amino acids because they are not limiting to protein synthesis (Coulson and Herbert, 1974; Coulson and Hernandez, 1968). In adult passerine birds, leucine is considered an essential amino acid whereas glycine is not (Griminger and Scanes, 1986; Murphy and King, 1986). In light of the above, and assuming that sparrows, like chickens, efficiently absorb leucine and glycine (Chung and Baker, 1992; Tasaki and Takashi, 1966), we tested the prediction that exogenous glycine will be oxidized more rapidly than leucine.

(3) Fatty acids. Given that exogenous FAs are differentially allocated among tissues (Babayán, 1987; McCue et al., 2009; Mu and Hoy, 2000) and that endogenous FAs are mobilized at different rates according to their physical structure, namely chain length and degree of unsaturation (Price et al., 2008), we predicted that exogenous FAs would be oxidized at different rates. When Jones et al. (Jones et al., 1985) examined whether oxidation rates of exogenous, 18-carbon FAs in humans were dependent on their degree of unsaturation, they found that although absorption efficiency of exogenous oleic and linoleic acid was similar (~98%), the cumulative oxidation of oleate was approximately 40% higher than linoleate. In addition, McCloy et al. (McCloy et al., 2004) found the cumulative oxidation of exogenous oleate and linoleate in humans to be 62% higher than exogenous linoleic acid. Assuming that absorption efficiencies of palmitic, stearic and oleic acids are similar to one another, as in chickens (~84%) (Hurwitz et al., 1973), we tested the prediction that sparrows oxidize longer, unsaturated FAs (e.g. oleic acid) more rapidly than their shorter or more saturated counterparts (e.g. palmitic and stearic acids).

## MATERIALS AND METHODS

### Animals

In 2008 and 2009 house sparrows, *Passer domesticus* L. ( $N=60$ ;  $25.3 \pm 2$  g; mean  $\pm$  s.d.) were captured with mist nets at Midreshet Ben-Gurion, Israel. The birds were banded with uniquely numbered aluminum or plastic leg bands and quarantined in a large, permanent outdoor aviary (4 m  $\times$  3 m  $\times$  2 m; length  $\times$  width  $\times$  height), where they were fed a diet of mixed millet seeds (approx. 12% protein and 5% lipid dry mass) (Williams and Ternan, 1999) and provided with tap water *ad libitum* for a minimum of 45 days. Crushed chicken egg shells, vitamin-supplemented water, and fresh lettuce were also provided once a week. Males and females were housed together, but reproduction was not observed.

At least one month before experiments, the birds were administered two deworming treatments 1 week apart to eliminate intestinal parasites that might influence oxidative dynamics of tracers. Birds were gavaged with an oral dose of Ivermectin ( $220 \mu\text{g kg}^{-1}$  in 0.5 ml water), followed a week later by a dose of Fenbendazole ( $30 \text{ mg kg}^{-1}$  in 0.5 ml water). After deworming, the sparrows were transferred into neighboring, smaller outdoor aviaries (1.5 m  $\times$  1.5 m  $\times$  2.5 m; length  $\times$  width  $\times$  height) with 8–12 individuals in each.

### Metabolic rates

Sparrows with full crops were removed from the aviaries and weighed to  $\pm 0.1$  g. Rates of oxygen consumption ( $\dot{V}_{\text{O}_2}$ ) and carbon dioxide production ( $\dot{V}_{\text{CO}_2}$ ) were then measured every 30 min between

10:00–15:00 h at  $24 \pm 1^\circ\text{C}$  ( $N=8$  females;  $N=14$  males) by open-flow, indirect calorimetry using the multiplexing respirometry system (Qubit Systems, Kingston, Ontario, Canada) previously described by Marom et al. (Marom et al., 2006).

$\dot{V}_{\text{O}_2}$  and  $\dot{V}_{\text{CO}_2}$  were calculated as  $\text{ml gas min}^{-1}$  using Eqns 1 and 2:

$$\dot{V}_{\text{O}_2} = \dot{V}_{\text{E}} \left( \frac{F_{\text{iO}_2} - F_{\text{eO}_2}}{1 - F_{\text{iO}_2}} \right), \quad (1)$$

$$\dot{V}_{\text{CO}_2} = \dot{V}_{\text{E}} (F_{\text{iCO}_2} - F_{\text{eCO}_2}), \quad (2)$$

where  $\dot{V}_{\text{E}}$  is the mass flow of gas passing through the metabolic chamber in  $\text{ml min}^{-1}$ ,  $F_{\text{iO}_2}$  and  $F_{\text{iCO}_2}$  represent the fractional concentrations of  $\text{O}_2$  and  $\text{CO}_2$  entering the metabolic chambers, and  $F_{\text{eO}_2}$  and  $F_{\text{eCO}_2}$  represent the fractional concentrations of  $\text{O}_2$  and  $\text{CO}_2$ , respectively, exiting the metabolic chambers.

The allometric relationships between body mass and metabolic rate of birds were characterized by fitting data to Eqn 3:

$$y = r M_b^z, \quad (3)$$

where  $y$  is the rate of oxygen consumption ( $\dot{V}_{\text{O}_2}$ ;  $\text{ml O}_2 \text{ min}^{-1}$ ) or carbon dioxide production ( $\dot{V}_{\text{CO}_2}$ ;  $\text{ml CO}_2 \text{ min}^{-1}$ ),  $M_b$  is body mass in grams, and  $r$  and  $z$  are an empirically determined coefficient and a mass exponent, respectively. Analysis of covariance (ANCOVA) was used to test for differences in metabolic rates between sexes.

### $^{13}\text{CO}_2$ analyses and calculations

For each round of isotope tracer measurements, four sparrows with full crops were selected from the communal aviaries at between 10:00 and 14:00 h. Background  $^{13}\text{CO}_2$  levels were determined by placing birds in 800 ml metabolic chambers at  $24 \pm 1^\circ\text{C}$  with dry,  $\text{CO}_2$ -free air pumped through each chamber at a rate of  $150\text{--}200 \text{ ml min}^{-1}$ , a flow rate that allowed  $F_{\text{eCO}_2}$  in the metabolic chambers to reach slightly less than 1% (Buyse et al., 2004). After 15 min, 10 ml of excurrent gas was collected by drawing the volume into a syringe at a rate of approximately  $60 \text{ ml min}^{-1}$ . The syringes were emptied into glass Exetainer vials (Labco, High Wycombe, Buckinghamshire, UK) previously flushed with helium.

After baseline  $\text{CO}_2$  samples had been collected, each bird was gavaged with 20 mg of one of seven isotopically labeled molecules (i.e. D-[1- $^{13}\text{C}$ ]glucose, 98–99%; D-[1- $^{13}\text{C}$ ]fructose, 99%; L-[1- $^{13}\text{C}$ ]leucine, 99%; [1- $^{13}\text{C}$ ]glycine, 99%; [1- $^{13}\text{C}$ ]palmitic acid, 99%; [1- $^{13}\text{C}$ ]stearic acid, 99%; [1- $^{13}\text{C}$ ]oleic acid, 99%; Cambridge Isotope Laboratories, Andover, MA, USA) suspended in 200  $\mu\text{l}$  of sunflower seed oil using a 15-g silicon-tipped polyethylene feeding tube (FTP-15-78; Instech Solomon, Plymouth Meeting, PA, USA) attached to a 1.0 ml syringe. Glucose was administered as suspension in sunflower seed oil or as a 200  $\mu\text{l}$  aqueous solution. Tracers were administered in a random order.

After administration, the birds were returned to the metabolic chambers and exhaled  $\text{CO}_2$  samples were taken at 15-min intervals over the next 2 h. Four sparrows were used twice in the breath tests, but none of them received tracers belonging to the same class of molecules twice. Prior to administering a second tracer to the birds, we confirmed that 30 days provided a sufficient washout period for the  $^{13}\text{CO}_2$  from the first tracer, and that the background  $^{13}\text{CO}_2$  in the breath of previously dosed individuals was not significantly different from that of untreated ones.

We also determined the shelf-life of isotopically enriched breath samples. Results indicated that, after 6 weeks ( $\sim 1000$  h) of storage at room temperature,  $^{13}\text{C}$  enrichment had not changed significantly. Nevertheless, all isotopic analyses were done within 4 weeks of collection.

The carbon isotope composition of the  $\text{CO}_2$  in the vials was quantified using gas source isotopic ratio mass spectrometry (GS-IRMS) through a Gas Bench II interface (Thermo-Fisher Scientific, Waltham, MA, USA). The  $\delta^{13}\text{C}_{\text{CO}_2}$  in each gas sample was calculated according to the Craig equation (Craig, 1957):

$$\delta^{13}\text{C}_{\text{‰PDB}} = \left( \frac{(^{13}\text{C}/^{12}\text{C})_{\text{sample}} - (^{13}\text{C}/^{12}\text{C})_{\text{std}}}{(^{13}\text{C}/^{12}\text{C})_{\text{std}}} \right) 10^3, \quad (4)$$

where  $^{13}\text{C}/^{12}\text{C}$  is the ratio ( $R$ ) of the heavy and the light carbon isotopes in the sample, compared with the ratio in a standard. Our standard was Pee Dee Belemnite (PDB) with a value of  $R_{\text{PDB}}=0.01112329$ . We determined the precision of measurements to be 0.1‰.

Since  $\delta^{13}\text{C}_{\text{‰PDB}}$  is neither an SI unit nor an appropriate metric for isotope tracer studies (Slater et al., 2001; Wetzel, 2005), we transformed  $\delta^{13}\text{C}$  to atom percent ( $\text{AP}^{13}\text{C}$ ) using Eqn 5 (Slater et al., 2001).

$$\text{AP}^{13}\text{C} = 10^2 \left( \frac{1}{\left( \frac{\delta^{13}\text{C}_{\text{PDB}}}{10^3} + 1 \right) R_{\text{PDB}}} + 1 \right)^{-1}. \quad (5)$$

In order to correct for background isotopic signatures and to avoid reporting small percentages, the atom fraction excess of the tracer ( $y \times 10^6 \text{ AFE}^{13}\text{C}$ ) was calculated using Eqn 6 (Slater et al., 2001):

$$y \times 10^6 \text{ AFE}^{13}\text{C} = ((\text{AP}^{13}\text{C})_{\text{E}} - (\text{AP}^{13}\text{C})_{\text{B}}) 10^3, \quad (6)$$

where  $(\text{AP}^{13}\text{C})_{\text{B}}$  and  $(\text{AP}^{13}\text{C})_{\text{E}}$  refer to background and enriched values, respectively. The instantaneous rates of tracer oxidation ( $T$ ;  $\text{nmol min}^{-1}$ ) were calculated using the following modified Fick equation:

$$T = \left( \frac{\dot{V}_{\text{CO}_2} \left( \frac{y \times 10^6 \text{ AFE}^{13}\text{C}}{10^2} \right)}{k} - 10^3 \right) (\theta \text{BRF})^{-1}, \quad (7)$$

where BRF is the bicarbonate retention factor (i.e. a correction factor that accounts for the loss of  $^{13}\text{C}$  tracer into the circulating bicarbonate pool) for birds [0.86 (Tabiri et al., 2002a)],  $\theta$  is the number of isotopically enriched atoms per tracer molecule, and  $k$  is the volume of  $\text{CO}_2$  (ml) produced per mg of tracer oxidized (Table 1). The value of  $k$  was calculated for each tracer molecule with the following equation that combines the stoichiometry of uric acid with the ideal gas law:

$$k = \frac{(C - (1.2N)) 22.4}{M}, \quad (8)$$

where  $M$  is the molar mass, 22.4 is the volume of one mole of gas at STP in liters, and  $C$  and  $N$  are the number of carbon and nitrogen atoms in each tracer molecule, assuming these birds convert all nitrogenous waste into uric acid (Griminger and Scanes, 1986).

We converted units to  $\mu\text{mol}$  and modeled the cumulative oxidation:

$$\left( \int_0^t f(x) dx \right) 10^{-3},$$

using a single-compartment, two-parameter exponential equation for amino acid and carbohydrate tracers:

$$f(t) = a(1 - e^{-bt}), \quad (9)$$

where  $t$  is time (in minutes) and  $a$  and  $b$  are empirically determined coefficients and exponents, respectively. The cumulative oxidation of fatty acids could not be modeled using an exponential model and was therefore described using a three-parameter, sigmoidal model of the following form:

$$f(t) = \frac{a}{1 + e^{-\left(\frac{t-x_0}{b}\right)}}, \quad (10)$$

where  $a$ ,  $b$  and  $x_0$  are empirically determined values.

StatView (SAS, Cary, NC, USA) was used for ANCOVA and SigmaPlot 11 (Systat, Chicago, IL, USA) was used for ANOVA, Holm–Sidak *post-hoc* tests,  $t$ -tests and curve fitting. Critical  $\alpha$  was set at 0.05, but Bonferroni-corrected  $P$ -values were used for multiple comparisons (e.g. instantaneous rates of tracer oxidation at all time points). The timing of peak oxidation was estimated by fitting instantaneous rates of tracer oxidation to sixth-order polynomials and solving for the local maximum. Values refer to means  $\pm$  s.d. unless indicated otherwise.

## RESULTS

The bilogarithmic relationships between  $\dot{V}_{\text{CO}_2}$  and  $M_b$  did not differ with sex (ANCOVA,  $P_{\text{slope}} < 0.0001$ ,  $P_{\text{intercept}} < 0.0001$ ). Rates of oxygen consumption and carbon dioxide production were described by the allometric equations  $\dot{V}_{\text{O}_2}$  (s.e.m.) =  $0.1296 (0.0368) M_b^{0.7627(0.0876)}$  and  $\dot{V}_{\text{CO}_2} = 0.1095 (0.0372) M_b^{0.7251(0.1048)}$ , and the mean respiratory exchange ratio was  $0.75 \pm 0.04$  ( $N=23$ ). The background  $\delta^{13}\text{CO}_2$  was  $-13.0 \pm 1.3\text{‰}$ , but 15 min after administration, the exhaled  $\text{CO}_2$  became significantly enriched in  $^{13}\text{C}$  (paired  $t$ -test; d.f.=63,  $P < 0.001$ ). Thereafter, the  $^{13}\text{CO}_2$  increased until reaching a peak; it then decreased to levels that remained significantly enriched over the background values during the next 2 h.

The rates at which different exogenous substrates were oxidized differed according to fuel type. The instantaneous rates of glycine oxidation were higher than leucine at all post-administration measurement times ( $t$ -tests, d.f.=15,  $P < 0.005$  at each time point; Fig. 1A). We found no significant differences between the instantaneous rates of oxidation of palmitic acid and oleic acid at any time point, but the instantaneous rates of oxidation of stearic acid differed significantly from the other two FAs at all time points ( $t$ -tests, d.f.=15,  $P < 0.001$  at all time points; Fig. 1B). The instantaneous rates of oxidation of glucose in oil, aqueous glucose, and fructose were generally similar, although oxidation rates of aqueous glucose was significantly higher than the other two carbohydrate treatments at 15 and 30 min following administration (two-way ANOVA followed by Holm–Sidak *post-hoc* pairwise comparisons,  $P < 0.001$ ; Fig. 1C).

The timing of peak  $^{13}\text{CO}_2$  production depended on the type of fuel. In general, peak  $^{13}\text{CO}_2$  production occurred earlier following administration of amino acid (20–46 min) and carbohydrate (24–34 min) than the FA (57–74 min; Table 1) tracers. The magnitude of peak  $^{13}\text{CO}_2$  production rate was also dependent on fuel type. Mean peak oxidation rates were  $2.02 \text{ mmol min}^{-1}$  and  $0.35 \text{ mmol min}^{-1}$  for glycine and leucine, respectively (Table 1). Mean peak rates of carbohydrate tracers were lower than those of amino acids but higher than those of FAs (Table 1). The maximum rate of aqueous glucose oxidation differed significantly from

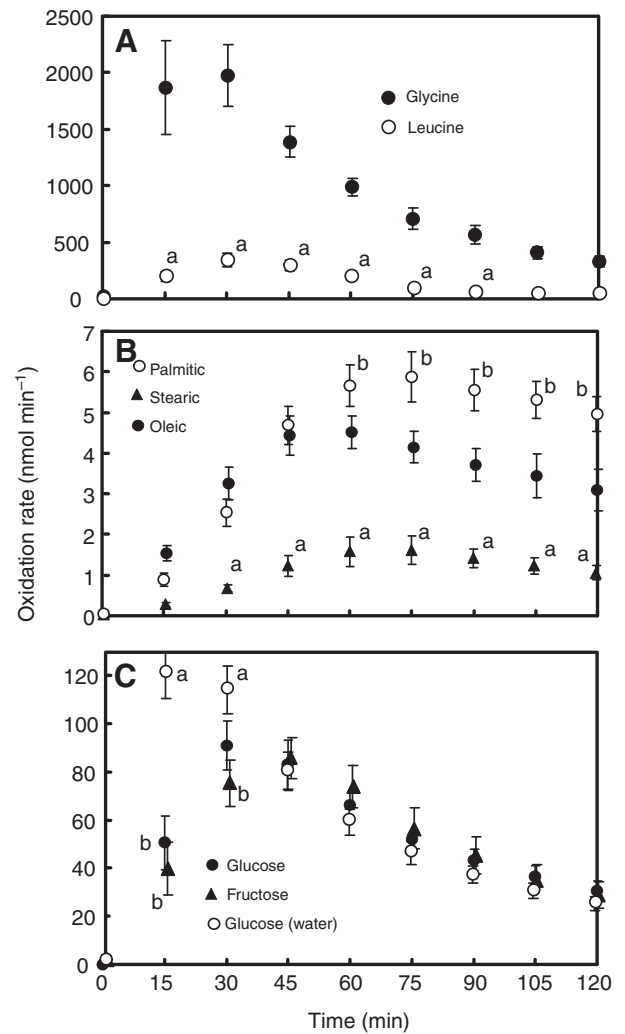


Fig. 1. Instantaneous rates of oxidation of seven exogenous metabolic fuels fed to postprandial sparrows: (A) amino acid, (B) fatty acids, (C) carbohydrates. Symbols are slightly offset for clarity. Different lower case letters indicate significant differences according to Holm–Sidak pairwise comparisons, or  $t$ -tests in the case of the amino acids. Values are mean  $\pm$  1 s.e.m.

fructose and glucose administered in oil (two-way ANOVA followed by Holm–Sidak *post-hoc* pairwise comparisons,  $P < 0.001$ ). Mean peak rates of  $^{13}\text{CO}_2$  production following administration of the FA tracers were two orders of magnitude less than those of amino acids (Table 1), and were  $6.0$ ,  $4.6$  and  $1.6 \text{ mmol min}^{-1}$  for palmitic, oleic and stearic acid, respectively.

Although the absolute variance in peak  $^{13}\text{CO}_2$  production differed widely among the different tracers, the coefficients of variation (CV) of mean peak  $^{13}\text{CO}_2$  production were less variable, ranging from 0.22 and 0.67 (Table 1). The CVs of stearic acid, which were the highest of all the tracers, were only significantly greater than those of oleic acid and glucose (Kruskal–Wallis ANOVA on ranks followed by Dunn's pairwise comparisons,  $P < 0.05$ ).

The cumulative tracer oxidation of carbohydrate and amino acid tracers were accurately modeled using two-parameter exponential equations. However, the cumulative oxidation of the three FAs showed clear logistic patterns and were therefore described using a three-parameter logistic equation (Table 1). Amino acid tracers were oxidized more extensively than the other classes of metabolic fuels

Table 1. The net volume of CO<sub>2</sub> produced per milligram of tracer oxidation in house sparrows

	<i>K</i> (ml CO <sub>2</sub> mg <sup>-1</sup> )	Time of peak (min)	Max. rate (nmol min <sup>-1</sup> )	CV at peak (s.d./mean)	<i>a</i>	<i>b</i>	<i>x</i> <sub>0</sub>	<i>F</i> <sup>2</sup>
Palmitic acid	1.398	74±7	6±1.9	0.31	0.8565±0.0696	21.0591±3.2641	69.1338±5.1086	0.8620
Oleic acid	1.427	57±7	4.6±1.0	0.22	0.6527±0.0453	21.4630±3.3037	59.9262±4.7000	0.8484
Stearic acid	1.417	74±8	1.6±1.1	0.67	0.2222±0.0316	20.4461±5.7844	68.4483±8.8425	0.6522
Glycine	0.229	24±4	2021±869	0.43	189.9802±16.3025	0.0224±0.0049	–	0.6639
Leucine	0.82	34±4	351±107	0.31	31.5923±2.2843	0.0213±0.0038	–	0.7770
Glucose	0.746	35±4	93±25	0.27	13.9645±1.9645	0.0111±0.0025	–	0.8279
Glucose-water	0.746	20±3	122±29	0.24	12.2952±0.7824	0.0213±0.0033	–	0.8945
Fructose	0.746	46±5	84±25	0.3	16.8152±4.9869	0.0081±0.0035	–	0.7283

*k*, tracer oxidation; CV, coefficients of variation.

Values *a*, *b*, *x*<sub>0</sub> and *F*<sup>2</sup> are parameters of the cumulative tracer oxidation models.

(Fig. 2A), but a greater proportion of glycine (45.2±3.8% of dose) was oxidized than leucine (12.8±0.9% of dose; *t*-test, *P*<0.001). Exogenous carbohydrate tracers were oxidized less extensively than the amino acids although to a greater extent than the FAs (Fig. 2C). Although we found differences in the timing and magnitude of the peak rates of oxidation, the cumulative proportion of the carbohydrate tracers oxidized did not differ significantly among treatments. Exogenous FAs were oxidized less extensively than were amino acids or carbohydrates (Fig. 2B), but the proportion of tracers oxidized differed in the three FAs (ANOVA, *P*<0.001). The proportion of oxidized stearic acid was significantly lower (0.21±0.03%) than palmitic acid (0.73±0.06%; Holm–Sidak test, *P*<0.001), or oleic acid (0.63±0.06%; Holm–Sidak test, *P*<0.001).

## DISCUSSION

We found that house sparrows oxidized exogenous amino acids far more rapidly than carbohydrates or FAs. The fact that the sparrows quickly catabolized excess amino acids, was somewhat surprising, but may be due to several factors, including (1) amino acids are less efficient lipogenic precursors than carbohydrates which make up most of the diet of these birds, (2) birds lack specialized tissues for protein storage, and (3) net rates of protein synthesis may be negligible in well-nourished, adult endotherms (Hatch et al., 2002b; Johnson et al., 1999). The large differences between the oxidative kinetics of exogenous glycine and leucine supported our prediction that nonessential amino acids are more readily catabolized than essential amino acids; however, it is possible that these differences are also related to the differences in the molecular sizes of leucine and glycine. Although 0.27 mmol of glycine was administered, compared with 0.15 mmol of leucine, the cumulative amount of glycine oxidized (i.e. 177±15 nmol) was over six times that of leucine (i.e. 29±2 nmol), suggesting this outcome is probably not an artifact of the mass action of a greater number of glycine molecules. A study examining the relationship between dose and rates of leucine oxidation in humans reported that tracer oxidation only differed by 20% across a fourfold range of doses (Cortiella et al., 1988).

It is probable that gut mucosal cells were responsible for some of the observed differences in exogenous leucine and glycine oxidation. Several studies of mammals have compared the fates of intravenous and oral doses of leucine, permitting researchers to estimate that between 17–41% of exogenous leucine is oxidized immediately (i.e. first-pass oxidation) upon absorption by splanchnic tissues (Cortiella et al., 1988; Crenn et al., 2000; Hoerr et al., 1991; Istfan et al., 1988; Luiking et al., 2005; Metges et al., 2000; Stoll et al., 1998; Yu et al., 1990). Although less is known about rates of first-pass glycine oxidation by splanchnic tissues, Daenzer et al. (Daenzer et al., 2001) suggested that in humans, as much as half the exogenous glycine may be lost to first-pass oxidation.

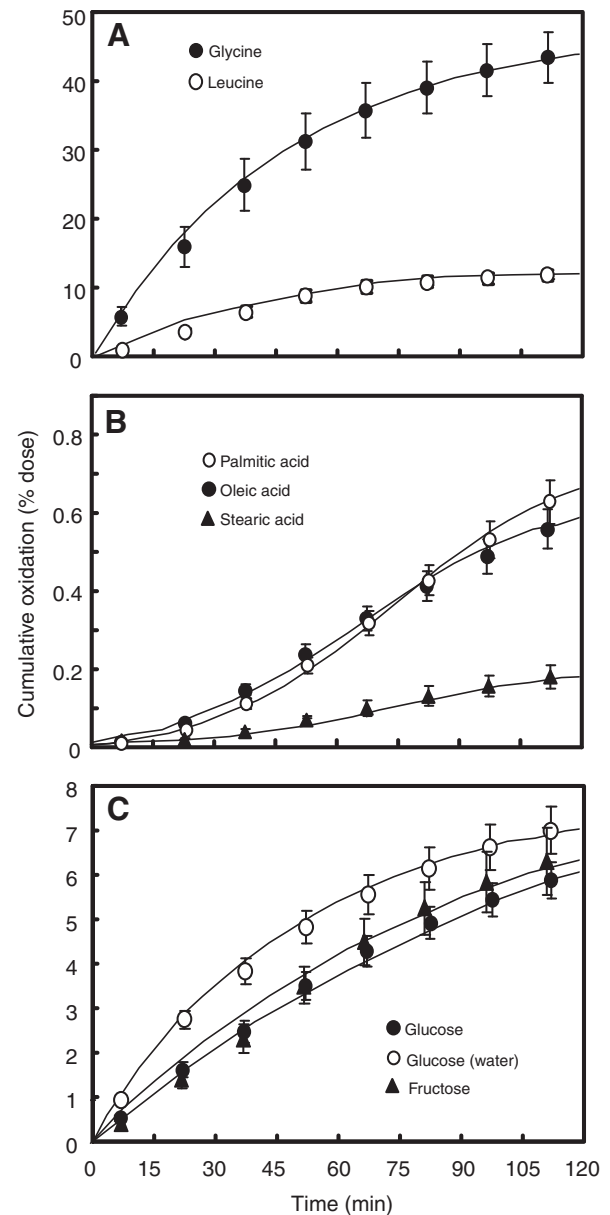


Fig. 2. Cumulative tracer oxidation in postprandial sparrows. Curves for amino acids (A) and carbohydrates (C) are described using a two-parameter exponential equation (Eqn 9) whereas FAs (B) are described using a three parameter logistic equation (Eqn 10). Parameters are presented in Table 1. Values are means ± s.e.m. of cumulative tracer oxidation and are slightly offset for clarity.

Fatty acids were oxidized at the lowest rates and to a lesser extent than the other tracers examined. If we assume that sparrows, like chickens, absorb common long-chain FAs with similar efficiencies (Hurwitz et al., 1973), we may conclude that longer chain saturated FAs (e.g. stearic) were more refractory to immediate oxidation than shorter chain FAs (e.g. palmitic acid) or unsaturated FAs (e.g. oleic acid). This possibility is also supported by the fact that granivorous birds consume large amounts of medium chain FAs, but do not retain them in significant levels in their tissues (Conway et al., 1994; McCue et al., 2009; Pierce et al., 2005; Zar, 1977).

Carbohydrates are considered to be one of the most metabolically accessible fuels for vertebrates (Kleiber, 1975; Navarro and Gutierrez, 1995; Wang et al., 2006), and are thought vital for sustaining the comparatively high metabolic demands of small mammals and birds (Cruz-Neto and Jones, 2006; Karasov and Cork, 1994; McWhorter et al., 2006; McWhorter et al., 2004; Voigt and Speakman, 2007). Although *in vitro* studies have demonstrated that sparrows are capable of comparatively high rates of mediated and paracellular absorption of monosaccharides (Caviedes-Vidal and Karasov, 1996; Chediack et al., 2001), until now we could only speculate about the rates at which exogenous sugars were oxidized *in vivo*.

The results of this study suggest that sparrows may not be capable of oxidizing exogenous carbohydrates as rapidly as nectarivores (Welch et al., 2006; Welch et al., 2008; Welch and Suarez, 2007), although it is difficult to make direct comparisons among these animals because the sparrows were digesting a mixed diet rather than just sugar. A comparison of exogenous glucose oxidation in exercising humans and resting sparrows, which have similar mass specific  $\dot{V}_{CO_2}$  ( $0.0371 \text{ ml CO}_2 \text{ min}^{-1} \text{ g}^{-1}$  [Jentjens et al., 2004] versus  $0.0458 \text{ ml CO}_2 \text{ min}^{-1} \text{ g}^{-1}$ ) indicates that exercising humans fed large amounts of dissolved glucose were able to oxidize exogenous glucose at a rate nearly twice that of the sparrows [i.e.  $10.5 \text{ ng g}^{-1} \text{ min}^{-1}$  (this study) versus  $17.3 \text{ ng g}^{-1} \text{ min}^{-1}$  (Jentjens et al., 2004)] in 2 h. Again it is notable that, unlike humans, sparrows in this study were digesting a meal of mixed composition, resulting in a situation where different substrates may be competing for passive uptake.

The maximum rate of exogenous glucose oxidation was significantly higher, and the time required for peak oxidation was shorter, when glucose was administered dissolved in water than when it was administered suspended in oil. Nevertheless, over the 120-min period, the cumulative amount of glucose oxidation did not differ between these two treatments, failing to support our prediction that more exogenous glucose would be oxidized when administered in an aqueous solution. We speculate that aqueous glucose may have been initially oxidized at a higher rate because it was more likely to be absorbed across the intestine by passive, rather than mediated means (*sensu* Caviedes-Vidal and Karasov, 1996; Chediack et al., 2001).

Because the tracers we used were not chemically integrated into the diet, it is possible that they were absorbed and oxidized more rapidly than if they had been integrated into larger, more complex molecules (e.g. starch, lipids, proteins, etc.). Consequently, we were unable to distinguish between the proportions of  $CO_2$  derived from endogenous and exogenous sources as has been done in postprandial pythons (Starck et al., 2004). A method for producing egg proteins with homogeneously integrated  $^{13}C$ -labeled amino acids was developed by Geboes et al. (Geboes et al., 2004), but a cost-effective method for creating comparatively large amounts of structured lipids (*sensu* Babayan, 1987) containing  $^{13}C$ -labeled FAs or highly enriched  $^{13}C$  starches remain unavailable.

Overall, the results of this study provide compelling evidence that  $^{13}CO_2$  breath testing can be used to track the oxidative fates of important physiological fuels in a dietary generalist. Although we only examined the rates at which healthy, adult sparrows oxidized seven metabolic tracers, our results lead to as many questions as they answer. For example, in addition to the need to examine how these observed patterns vary among species (Schwenk et al., 2009), below we suggest several ideas where breath testing may be fruitfully used in research. Because wild birds are rarely in a steady nutritional state, it is important to investigate how rapidly growing individuals or individuals recovering from negative protein balance oxidize exogenous amino acids. Moreover, future studies that use a wider diversity of FAs than we did, such as medium chain FAs (e.g. caprylic acid, capric acid and lauric acid) and highly polyunsaturated FAs (e.g. linoleic acid,  $\alpha$ -linoleic acid and mead acid) will be useful to examine how the chain length and degree of unsaturation influences the differential disposal of exogenous FAs. Studies comparing the fates of metabolic tracers that are integrated into macromolecules may help determine the degree to which oxidative kinetics of purified tracers can proxy for materials in natural diets. Coincidentally, examinations designed to quantify the sensitivity of oxidative kinetics to tracer dose will be helpful to facilitate interspecific comparisons.

Although the past few years have witnessed an increase in the use of breath testing in nectarivorous endotherms (Voigt et al., 2008a; Voigt et al., 2003; Voigt et al., 2008b; Voigt and Speakman, 2007; Welch et al., 2006; Welch et al., 2008; Welch and Suarez, 2007), we suggest that breath testing can be used to study a much wider range of animal models. The approaches we outline here offer a robust methodological framework that can be applied to all types of animals and be tailored to test a variety of hypotheses. We anticipate breath testing becoming a powerful tool for physiological ecologists and evolutionary physiologists wishing to examine how factors such as ontogeny, season, dietary habituation, exercise regime, ambient temperature, hydration levels, hypometabolism and nutritional stress influence how animals differentially rely on exogenous fuels.

#### LIST OF SYMBOLS AND ABBREVIATIONS

$AP^{13}C_B$	atom percent of heavy isotope in the breath before tracer administration
$AP^{13}C_E$	atom percent of heavy isotope in the breath following tracer administration
BRF	bicarbonate retention factor (estimated)
$d$	mass of tracer administered (mg)
$F_{eCO_2}$	fractional concentration of carbon dioxide in excurrent gas stream
$F_{eO_2}$	fractional concentration of oxygen in excurrent gas stream
$F_{iCO_2}$	fractional concentration of carbon dioxide in incurrent gas stream
$F_{iO_2}$	fractional concentration of oxygen in incurrent gas stream
$k$	volume of carbon dioxide produced per mg of tracer oxidized (ml)
$M$	molar mass of tracer ( $\text{g mol}^{-1}$ )
$M_b$	body mass (g)
$t$	time (min)
$T$	instantaneous rate of tracer oxidation ( $\text{nmol min}^{-1}$ )
$\dot{V}_{CO_2}$	rate of carbon dioxide production ( $\text{ml min}^{-1}$ )
$\dot{V}_{O_2}$	rate of oxygen consumption ( $\text{ml min}^{-1}$ )
$\dot{V}_E$	flow rate of gas corrected for standard temperature and pressure ( $\text{ml min}^{-1}$ )
$y_{10^6 AFE}^{13}C$	atom fraction excess of heavy isotope in a sample
$\delta^{13}C_{\text{‰PDB}}$	ratio of heavy and light carbon isotope in a sample compared to Pee Dee Belemnite
$\theta$	number of heavy atoms in a given tracer molecule

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