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Hummingbirds can fuel expensive hovering flight completely with either exogenous glucose or fructose

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Summary

1. Hummingbirds have specialized on a diet consisting almost exclusively of a mixture of sucrose, glucose and fructose found in floral nectar. Previous studies have shown that hummingbirds can fuel energetically expensive hovering flight almost exclusively using recently ingested sucrose. However, the relative capacities for the direct utilization of glucose and fructose by hovering hummingbirds remain unknown.

2. ^{13}C-enriched solutions of glucose and fructose were fed to ruby-throated hummingbirds (*Archilochus colubris*) separately. Along with simultaneous measurements of gas exchange during hovering we collected exhaled breath samples using feeder-mask respirometry and analysed these to determine the isotopic signatures of exhaled carbon dioxide. We found that hovering hummingbirds transition from exclusively oxidizing endogenous fatty acids when fasted, to oxidizing newly ingested carbohydrates when given access to either glucose or fructose solutions. We then switched hummingbirds to the respective unlabelled solutions of glucose or fructose to estimate carbohydrate turnover kinetics.

3. During the period of availability of enriched solutions, the percentage of metabolism supported by exogenous sugar increased from 0% to near 100% in some individuals. On average, hummingbirds fuelled 81% and 88% of their metabolism during hovering flight with exogenous glucose and fructose, respectively.

4. The amount of energy ingested, fractional turnover of ingested sugars in the pool of actively metabolized substrates, amount oxidized, energy expended and proportion of hovering metabolism supported by each hexose were all similar between glucose and fructose.

5. By foraging frequently and fuelling hovering flight directly with ingested monosaccharides hummingbirds avoid the energetic tax associated with the cost of synthesis of fats from these sugars prior to their oxidation. Remarkably, hovering hummingbirds are able to utilize fructose and glucose equally, a physiological feat which no mammals are thought to match, and one that suggests novel physiological capacities for the oxidation of fructose by active muscle tissues in hummingbirds. The data presented here indicate hummingbirds enhance net energy intake though specialization of diet, behaviour, and, uniquely, metabolic physiology.

Key-words: *Archilochus colubris*, respirometry, energetics, fructose, glucose, fuel use, stable isotope, sucrose

Introduction

When sustaining true hovering flight hummingbirds have some of the highest mass-specific metabolic rates among vertebrates (Suarez 1992). Hovering flight enables hummingbirds to access energy-rich nectar in flowers, and it is the sugar in floral nectar that accounts for almost all of the energy they ingest (Powers & Nagy 1988).

Nectars ingested by hummingbirds are composed almost entirely of sucrose, glucose and fructose (Percival 1961; Martinez del Rio 1990; Baker, Baker & Hodges 1998). When ingested, sucrose, glucose and fructose are

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assimilated in the intestines with equally high efficiency (>97%; Martinez del Rio 1990). Sucrose must be hydrolysed to glucose and fructose in the gut by the membrane-bound enzyme sucrase before the resulting monosaccharides can be transported into intestinal cells (McWhorter *et al.* 2006).

Given their sugar-rich diet and high metabolic rate during flight, it is perhaps not surprising that hummingbirds can fuel hovering exclusively via the oxidation of recently ingested sucrose (Suarez *et al.* 1990; Welch *et al.* 2006; Welch & Suarez 2007; Suarez, Herrera & Welch 2011). This is in contrast to other avian taxa that appear to rely primarily or exclusively on fatty acid oxidation to fuel flight (e.g. Tucker 1968, 1972; Torre-Bueno & Larochelle 1978; Hudson & Bernstein 1983; Ward *et al.* 2001; Bundle, Hansen & Dial 2007). These species, however, are not nectarivorous. The dearth of simple sugars in their diets likely do not afford them the opportunity to rely on dietary sugars to fuel flight. Instead, these birds rely on the fuel source that offers greatest energy density, and thus mass-savings (Jenni & Jenni-Eiermann 1998; Guglielmo 2010). It seems likely that the ability to minimize mass gain by storing energy as fat, rather than glycogen, is a primary reason why hummingbirds also exhibit a reliance on fatty acids as a fuel during fasting (Suarez *et al.* 1990). While hummingbirds require small amounts of protein and micronutrients from insects and pollen for long term survival (Brice & Grau 1991; López-Calleja, Fernández & Bozinovic 2003; Nicolson & Fleming 2003), amino acid catabolism does not contribute significantly to the fuelling of typical foraging behaviour.

Unlike hummingbirds, humans and most mammals can support only a small proportion (≤30%) of exercise metabolism by the oxidation of recently ingested sugars (e.g. Adopo *et al.* 1994; Jeukendrup *et al.* 1997, 2006; Jentjens *et al.* 2004). Limitations on the reliance of exogenous sugars as a fuel can (in comparison to hummingbirds) be explained, in part, by constraints on the rate of absorption of carbohydrates across the intestine (Jentjens *et al.* 2004). However, muscle tissue perfusion, muscle fibre membrane transport activity, and flux through catabolic pathways also likely limit the use of circulating sugars in humans and other mammals (Rose & Richter 2005). While ingestion of mixtures of glucose and fructose enable greater reliance on exogenous carbohydrates as a fuel source during exercise compared with ingestion of either monosaccharide alone, reliance on recently ingested fructose is more limited than on glucose in humans (Adopo *et al.* 1994; Massicotte *et al.* 1994; Jentjens *et al.* 2004). As much as 50% of ingested fructose must first be converted to glucose in the liver before it can be used as an oxidative substrate for muscle fibres in humans (Delarue *et al.* 1993). Additionally, rates of uptake (Kristiansen *et al.* 1997) and oxidation or conversion to lactate or glycogen in muscle fibres (Zierath *et al.* 1995) are much lower for fructose than for glucose.

While previous studies on hummingbirds demonstrated a remarkable capacity for the oxidation of exogenous carbohydrates derived from newly ingested sucrose solutions (Welch *et al.* 2006; Welch & Suarez 2007; Suarez, Herrera & Welch 2011), these studies could not quantify relative capacities for the oxidation of component monosaccharides glucose or fructose. Previous research has also demonstrated that members of another highly aerobic, nectarivorous group of flying vertebrates, glossophagine nectar bats, possesses a similarly enhanced capacity to support hovering flight with oxidation of newly ingested sugars (Voigt & Speakman 2007; Welch, Herrera & Suarez 2008; Suarez, Herrera & Welch 2011). A study by Voigt & Speakman (2007) demonstrated that restrained bats that had recently flown exhaled carbon dioxide derived primarily from the oxidation of recently ingested carbohydrates, regardless of whether those ingested carbohydrates were glucose, fructose or the disaccharide sucrose. Relative reliance on fructose and glucose was generally similar and these results indirectly supported the hypothesis that nectar bats were equally adept at using either fructose or glucose to directly fuel flight muscle metabolism (Voigt & Speakman 2007).

Because hummingbirds feed on diets equally rich in glucose and fructose (Baker, Baker & Hodges 1998), and because they are able to so quickly and completely utilize carbohydrates in ingested sucrose solutions to fuel hovering, we hypothesized that they possess the capacity to mobilize and oxidize glucose and fructose equally rapidly and extensively during exercise.

To test this hypothesis, we employed ^{13}C-breath testing (Welch *et al.* 2006; Welch & Suarez 2007; McCue 2011) combined with open, flow-through feeder mask respirometry (Welch 2011) on ruby-throated hummingbirds (*Archilochus colubris*) offered one of three artificially or naturally isotopically labelled sugar solutions: ^{13}C-1-D-glucose, ^{13}C-1-D-fructose or sucrose from cane sugar. We measured the stable carbon isotope ratio of exhaled carbon dioxide ($\delta^{13}C_{breath}$) to determine respective reliance on exogenous (labelled) and endogenous fuels. We hypothesized that we would observe similar kinetics with respect to the timing and extent of a shift towards reliance on fructose, glucose or sucrose when hummingbirds fed during exercise. We predicted similar shifts in both the respiratory quotient ($RQ = \dot{V}_{CO_2}/\dot{V}_{O_2}$; from 0·7 to 1·0) as well as the isotopic signature of expired CO_2.

Additionally, we predicted hummingbirds would exhibit similar patterns of behaviour (e.g. proportion of time spent flying/feeding, energy ingestion rate) and similar time-energy budgets (i.e. hummingbirds would oxidize similar proportions of ingested energy) when either isocaloric fructose or glucose was offered.

Materials and methods

All protocols were approved by the University of Toronto's University Animal Care Committee.

We report $\delta^{13}C$ on a per mil (‰) basis relative to the international carbon standard, Vienna Pee Dee Belemnite (VPDB), Where

$$\delta^{13}C = \frac{(^{13}C/^{12}C)_{\text{sample}} - (^{13}C/^{12}C)_{\text{standard}}}{(^{13}C/^{12}C)_{\text{standard}}} \times 10^3 \quad \text{eqn 1}$$

All solid, liquid and gas samples were submitted to the Cornell University Stable Isotope Laboratory for the analysis of $^{13}C/^{12}C$ ratios by mass spectrometry. Samples were analysed on a Thermo Delta V Advantage isotope ratio mass spectrometer (Thermo Scientific, West Palm Beach, FL, USA) interfaced to a NC2500 elemental analyzer (solid/liquid samples) and Gas Bench II (gas samples).

EXPERIMENTAL PROTOCOL

Male *Archilochus colubris* ($n = 6$) were captured with a modified box trap (drop door trap) in Toronto, Ontario, Canada at the University of Toronto Scarborough (UTSC). Captive hummingbirds were housed individually in mesh enclosures measuring 60 × 60 × 60 cm at the UTSC vivarium. Once captive, birds were fed *ad libitum* on an 18% (w/v) solution of Nektar-Plus (Guenter Enderle, Tarpon Springs, FL, USA). This solution was made available to the hummingbirds on days preceding (and immediately following) fructose and glucose trials. The $\delta^{13}C$ value of this maintenance diet was $-20{\cdot}77 \pm 4{\cdot}69$‰ ($n = 13$; Table 1). When fructose and glucose trials were completed, the maintenance diet was switched to an 18% (w/v) mixture solution of 50% Nektar-Plus and 50% beet sucrose, 2 weeks before the commencement of sucrose trials. This mixed maintenance diet had a slightly more depleted isotopic signature, and we anticipated that this would result in the deposition of endogenous energy stores with an isotopic signature that was more distinct from the cane sugar offered during the trial period. The $\delta^{13}C$ value of this second maintenance diet was $-24{\cdot}31 \pm 0{\cdot}14$‰ ($n = 10$; Table 1). Live *Drosophila melanogaster* were given as a form of behavioural enrichment and nutritional supplement throughout. Flies were not available to the birds during trial periods. Birds were subjected to a 14-h light/10-h dark photophase cycle.

Experimental design and data collection were slightly modified from those described in Welch & Suarez (2007). Data collection was conducted in an acrylic enclosure measuring 128 cm length × 92 cm width × 93 cm height. A perch was available to the hummingbird and was placed on top of a balance (Model SCL-HP; Sable Systems International, Las Vegas, NV, USA) to monitor mass at 0·5 s intervals during data collection to a laptop computer for recording via an IM1 Promethion interface (Sable Systems International) using EXPEDATA software (v. 1.3.20; Sable Systems International). Data collection took place between August 2011 and June 2012 between 7.30 h and 12.00 h. Data collection began early in the morning, following the overnight fast. This ensured that there was clear temporal discrimination between endogenous energy stores and the newly available sugar. In addition, fasted hummingbirds are hungriest and most cooperative early in the morning (Suarez *et al*. 1990; Welch *et al*. 2006; Welch, Altshuler & Suarez 2007; Welch & Suarez 2007).

Table 1. Stable carbon isotope signatures $\delta^{13}C$ of maintenance diet, fructose, glucose and sucrose solutions used during experimental protocol (values are mean ± SEM)

Solution	$\delta^{13}C$ (‰, VPDB)	n
18% (w/v) solution of Nektar-Plus (1st maintenance diet for glucose and fructose experiments)	−20·77 ± 4·69	13
18% (w/v) solution of 50% Nektar-Plus + 50% beet sucrose (2nd maintenance diet for sucrose experiments)	−24·31 ± 0·14	10
1 M of 5% D-[1-^{13}C]fructose (0–150 min)	644·69 ± 19·17	8
1 M D-fructose (150–300 min)	−5·89 ± 10·84	8
1 M of 5% D-[1-^{13}C]glucose (0–150 min)	691·26 ± 41·95	6
1 M D-glucose (150–300 min)	−9·92 ± 1·68	6
1 M cane sucrose (0–150 min)	−12·03 ± 0·19	12
1 M beet sucrose (150–300 min)	−25·83 ± 0·13	12

VPDB, Vienna Pee Dee Belemnite C standard.

Following their overnight fast, one of three 1 M isotopically enriched solutions were offered for the first 150 min: 5% of D-[1-^{13}C]fructose, 99% (Cambridge Isotope Laboratories, Tewksbury, MA, USA) or 5% of D-[1-^{13}C]glucose, 99% (Cambridge Isotope Laboratories) or cane sucrose, 100% (Everyday Market, Canada). The $\delta^{13}C$ values for these three solutions were $644{\cdot}69 \pm 19{\cdot}17$‰ ($n = 8$; Table 1), $691{\cdot}26 \pm 41{\cdot}95$‰ ($n = 6$; Table 1) and $-12{\cdot}03 \pm 0{\cdot}19$‰ ($n = 12$; Table 1), respectively. Fructose and glucose solutions were equimolar, so that energy density would not be a confounding factor. However, the 1 M sucrose solution contained twice the energetic density of either monosaccharide solution. The concentration of the sucrose solution was chosen to facilitate comparison of behavioural variables and energy intake and expenditure with other published studies (Hainsworth 1973; Hainsworth & Wolf 1976; Gass, Romich & Suarez 1999; Nicolson & Fleming 2003; Fleming *et al*. 2004). The order of birds and treatment given (i.e. fructose or glucose) was randomized before the start of experiments. Data collection using birds offered the sucrose solution took place following all other treatments. Individual testing order was again randomized.

After 150 min, each solution was replaced with an identical, unlabeled solution: either D-fructose ($-5{\cdot}89 \pm 10{\cdot}84$‰, $n = 8$; Table 1; Sigma-Aldrich, Oakville, Ontario, Canada) or D-glucose ($-9{\cdot}92 \pm 1{\cdot}68$‰, $n = 6$; Table 1; Sigma-Aldrich) or beet sucrose ($-25{\cdot}83 \pm 0{\cdot}13$‰, $n = 12$; Table 1; New Foods, Charlotte, North Carolina, USA). These solutions were available for *c*. 150 min, such that each experimental period lasted a total of 300 min. Between trials (i.e. fructose, glucose or sucrose treatments), birds were returned to the vivarium for a minimum of 1 week and fed the maintenance diet. This ensured that labelled carbon could largely be cleared from their bodies and $\delta^{13}C$ breath values would be allowed to return to levels reflective of the maintenance diet before undergoing other acute experimental treatments.

RESPIROMETRY

Oxygen consumption and carbon dioxide production rates during hovering were obtained via mask respirometry (Bartholomew & Lighton 1986; Welch & Suarez 2007; Welch 2011). Expired air samples were obtained when hovering hummingbirds voluntarily inserted their heads into a plastic mask attached to feeder. This plastic mask was made from a disposable 10-mL luer lock syringe (BD, Franklin Lakes, NJ, USA) connected to Tygon® tubing leading to a nectar reservoir in a syringe pump (NE-500; New Era Pump Systems, Farmingdale, NY, USA). Tygon® tubing was attached to the side of the mask approximately halfway along its length through which incurrent air was drawn and delivered to the respirometry equipment (TurboFOX5; Sable Systems International).

In addition, an infrared (IR) emitter and detector were placed on either side of the front edge of the mask. Feeding duration was noted by the disruption of IR beam by the presence of bird's head in the mask which in turn triggered the syringe pump to deliver solution to the feeder at a set flow rate. Following pretrial testing, a flow rate of 500 μL min^{-1} was chosen as it maximized feeding

event durations and ensured no solution loss due to spillage. The combination of the IR detector and syringe pump permitted the calculation of the amount of solution ingested by the hummingbird for any feeding event as feeding duration multiplied by nectar flow rate. Before the commencement of experiments, the carbon dioxide analyser was calibrated with pure nitrogen gas (zero gas; Praxair, Mississauga, Ontario, Canada) and spanned with 0·25% CO_2 in nitrogen gas (Praxair). The oxygen analyser was calibrated with well-mixed ambient air drawn through the mask in the absence of a hummingbird. A thermoresistor probe was placed near the mask to record the ambient temperature in the plastic enclosure of the arena.

Flow rate through the mask was maintained at 1500 mL min^{-1}. The excurrent airstream was subsampled at ~800 mL min^{-1}. Subsampled air first passed through the TurboFOX5's water vapour meter module (TurboFOX5; Sable Systems International) which measured water vapour pressure. The air next passed through indicating Drierite (W.A. Hammond DRIERITE, Xenia, OH, USA) for the removal of water vapour before entering into the oxygen (fuel cell) and carbon dioxide (infrared) gas analysers (TurboFOX5; Sable Systems International). Drierite was preconditioned by exposure to ambient air prior to use to minimize the absorption of CO_2. Analogue voltage output from the IR detector, thermoresistor, oxygen and carbon dioxide analysers, flow meter, water vapour pressure and in-line barometric pressure sensors were recorded at 0·1 s intervals over the duration of the trial using EXPEDATA software (v. 1.3.20; Sable Systems International) and were reported to a laptop computer *via* on-board A/D converter through the serial output of the TurboFOX5.

The primary flow rate through the mask was corrected for the presence of water vapour as in Welch (2011; following Lighton 2008). Raw oxygen and carbon dioxide traces for each feeding event were used to determine the fractional concentration of oxygen depletion and carbon dioxide enrichment by first using drift correction and subtracting the traces with baseline values. These baseline values were determined by selecting two points, through linear extrapolation, directly before and after the feeding event being analysed. The peaks in the traces were then integrated over time to yield the difference in fractional concentration of oxygen consumed or carbon dioxide produced. Integrated volumes of oxygen consumed and carbon dioxide produced were calculated using standard equations (Withers 1977; Lighton 2008; Welch 2011). Rates of oxygen consumption and carbon dioxide production were determined by dividing the calculated volumes by the duration of the respective feeding event. Using these rates, respiratory quotient values ($= \dot{V}_{CO_2}/\dot{V}_{O_2}$) for any feeding event were determined by dividing the rate of carbon dioxide production ($\dot{V}_{CO_2}$) over the rate of oxygen consumption ($\dot{V}_{O_2}$).

STABLE CARBON ISOTOPE ANALYSIS OF EXPIRED CO_2

Exhaled gases were collected while the hummingbird was hover feeding at the respirometry mask downstream of the TurboFOX5 gas analysers. By sampling downstream of the gas analysers, we obtain gas samples without affecting calculated $\dot{V}_{O_2}$ and $\dot{V}_{CO_2}$. measurements. Because the samples contained a mixture of breath and ambient CO_2, $\delta^{13}C_{breath}$ was determined through the use of the two-part concentration-dependent mixing model adapted from Phillips & Koch (2002), such that:

$$\delta^{13}C_{breath} = [\delta^{13}C_{sample} - \delta^{13}C_{ambient}(f_a)]/(1 - f_a) \quad \text{eqn 2}$$

where $\delta^{13}C_{sample}$ is the $\delta^{13}C$ of air collected in the syringe. $\delta^{13}C_{ambient}$ is the $\delta^{13}C$ of the surrounding air (an average of samples taken from the mask at three points during the 300-min experiment; one before, one halfway and one at the end of the 300-minute period) when a hummingbird was absent at the mask. f_a is the fraction of CO_2 in the gas sample from ambient air. Immediately following CO_2 collection, gas samples were injected into pre-evacuated 12-mL Exetainer vials (Labco Limited, Buckinghamshire, UK) until a positive pressure was achieved. The samples were sent for stable isotope analysis to Cornell University, Stable Isotope Laboratory to determine [CO_2] (ppm) and $\delta^{13}C$ values. Samples with f_a values >0·7 were discarded from further analysis. This cut-off was determined through sensitivity analyses to reduce the 'noise' (ambient air) to 'signal' (breath) ratio in the data set.

CARBOHYDRATE OXIDATION KINETICS

To understand carbohydrate turnover kinetics, we assumed a single-compartment, first-order kinetics mixing model (Carleton, Bakken & Martínez del Rio 2006; Welch & Suarez 2007) and applied a nonlinear fit to $\delta^{13}C_{breath}$ values, separately, for the first 150 min (enriched solution) and for the last 150 min (unlabelled solution). The nonlinear fitting formula is:

$$\delta^{13}C_{breath}(t) = \delta^{13}C_{breath}(\infty) + [\delta^{13}C_{breath}(0) - \delta^{13}C_{breath}(\infty)]e^{-kt} \quad \text{eqn 3}$$

where $\delta^{13}C_{breath}(t)$ is the isotopic composition of exhaled CO_2 at time t, $\delta^{13}C_{breath}(0)$ is the isotopic composition of the carbon in expired CO_2 during the initial feeding event for the calculation of appearance kinetics and from the final feeding event prior to solution replacement for the calculation of disappearance kinetics, respectively, $\delta^{13}C_{breath}(\infty)$ is the asymptotic equilibrium isotopic composition of the carbon in expired CO_2, and k is the fractional rate of isotope incorporation into the pool of expired CO_2 (O'Brien, Schrag & Martínez del Rio 2000; Carleton & Martínez del Rio 2005; Carleton, Bakken & Martínez del Rio 2006; Welch & Suarez 2007). The subscript 'i' (for incorporation) and 'd' (for disappearance) are applied to k during the period of the experiment in which the enriched solution and unlabelled solution are available, respectively. Using equation 3, $\delta^{13}C_{breath}$ values were calculated every 5 min by solving for $\delta^{13}C_{breath}$ at the midpoint of the observed 5-min block (in minutes).

With these values, the proportion of expired CO_2 supported by exogenous, labelled carbohydrates (f_{exo}) for the 300 min can be calculated (Welch & Suarez 2007) for any 5-min block:

$$f_{exo} = (\delta^{13}C_{breath} - \delta^{13}C_{maintenance})/(\delta^{13}C_{acute} - \delta^{13}C_{maintenance}) \quad \text{eqn 4}$$

where $\delta^{13}C_{acute}$ is the $\delta^{13}C$ value of the relatively enriched solution, $\delta^{13}C_{maintenence}$ is the $\delta^{13}C$ value of endogenous fuels at time zero ($\delta^{13}C_{breath}$ (0) by using equation 3) during the first 150 min of the experiment and $\delta^{13}C_{maintenence}$ is the $\delta^{13}C$ value of the unlabelled solutions during the last 150 min of the experiment. Due to differences in the isotopic signatures among fructose, glucose and sucrose solutions, we report f_{exo} values rather than $\delta^{13}C_{breath}$, facilitating direct comparisons among treatments.

When a mole of glucose or fructose is oxidized, 6 moles of oxygen are consumed. For a mole of sucrose (fructose plus glucose), 12 moles of oxygen are consumed. Thus, the amount of enriched solution oxidized ($M_{enriched}$; in μmol) during each 5-min period may be estimated as:

$$M_{enriched} = [f_{exo}(M_{block}) \times 10^6]/n \quad \text{eqn 5}$$

where M_{block} is the amount of oxygen (in mol) consumed during that 5-min block (see time and energy budget calculation below) and n is 6 when fructose or glucose is oxidized or 12 when compo-

nent monosaccharides of sucrose are oxidized. The proportion of ingested solution oxidized was estimated by dividing the total molar amount metabolized ($M_{enriched}$) over the 300-min experiment by the molar amount of solution ingested over the first 150 min.

TIME AND ENERGY BUDGETS

Time budgets were assessed and used to derive total energy expenditures for each treatment group (see review in Goldstein 1988). Hummingbird activity was recorded using a digital video camera (Canon HD Vixia HF200; Canon Canada Inc., Mississauga, ON, Canada) for the entire duration (300 min) of each experiment. The activities were separated into 5-min blocks, with the first block starting when the hummingbird first fed at the mask. The volume of oxygen consumed during each 5-min block was determined by multiplying the durations of hovering or perching with each activity's respective mass-specific $\dot{V}_{O_2}$ and by multiplying estimates of hummingbird mass to the closest 5-min block. The amount of oxygen (in mL) was then converted to moles of O_2 at STP to derive M_{block} (the molar amount of oxygen consumed during each 5-min block; see above). Data on hovering metabolic rate ($MR_{block(hov)}$; mL O_2 h^{-1}) were collected simultaneously when breath samples were collected. While steady state forward flight at moderate speeds (5–10 m s^{-1}) incurs a lower metabolic cost than does hovering flight in small hummingbirds (Clark & Dudley 2010), the cost of low-speed forward flight (0–5 m s^{-1}) is not substantially different from that of hovering. The small size of the chamber used in our study constrained forward flight velocity in our hummingbirds. Because flight velocities were likely quite low, because the variation in metabolic cost between low-speed forward flight and hovering is minimal, and because good estimates of the metabolic cost of acceleration and deceleration are not available, we assume all flight behaviours incurred the same metabolic cost per time as hovering.

To obtain perching metabolic rate of hummingbirds, complementary mass specific measurements of oxygen consumption rate (in mL O_2 g^{-1} h^{-1}) and carbon dioxide production rate (in mL CO_2 g^{-1} h^{-1}) were taken for each hummingbird immediately after the experiment was completed. To accomplish this, the balance inside the arena was placed close to the mask, allowing the birds to feed voluntarily as they perched. No expired breath subsamples were collected, and the flow rate was maintained at 1500 mL min^{-1}. Five separate perch feedings from each bird were recorded, and the mean of these was used for energy budget calculations. Otherwise, all procedures were identical to those employed when hovering metabolic rate measurements were obtained.

Assuming hummingbirds oxidize fat and/or carbohydrate and that protein oxidation contributes little to overall metabolic function during foraging behaviour (Suarez *et al.* 1990; Welch, Altshuler & Suarez 2007), total energy expenditure for each 5-min block (E_{block}; in J) can be calculated as (following Welch & Suarez 2007):

$$\begin{aligned} E_{\text{block}} = \{&[(1 - \text{RQ}/0\cdot29] \times [h_{\text{oxygen(fat)}}] \\ &+ [(\text{RQ} - 0\cdot71)/0\cdot29] \times [h_{\text{oxygen(carb)}}]\} \\ &\times \{t_{\text{hov}}[\text{MR}_{\text{block(hov)}}/3600] \\ &+ t_{\text{perch}}[\text{MR}_{\text{block(perch)}}/3600]\} \end{aligned} \quad \text{eqn 6}$$

where RQ is the respiratory quotient for the feeding event closest to the 5-min block and is constrained to be between 0·71 and 1·0, $h_{oxygen(fat)}$ is the thermal equivalent of oxygen exchange when fat is the metabolic substrate (19·8 J ml^{-1}) (Brouwer 1957), $h_{oxygen(carb)}$ is the thermal equivalent of oxygen exchange when carbohydrates are the metabolic substrate (21·1 J ml^{-1}) (Brouwer 1957), t_{hov} and t_{perch} are the time spent on hovering and perching, respectively, for the 5-min block being analysed, and $MR_{block(hov)}$ and $MR_{block(perch)}$ are the metabolic rates of oxygen consumption during hovering and perching (mL O_2 h^{-1}), respectively, for the 5-min block being analyzed.

A generalized linear model (GLM) mixed-design ANOVA (a combination of a factorial ANOVA and a repeated measures ANOVA) was fitted to the data to examine the effect of time and treatment (sugar offered) on RQ and f_{exo} values. Only data from the first 40 min of the experiment (period beginning 40 min after the first feeding of enriched solution) was used to build the model for two reasons: (i) The greatest change in RQ and f_{exo} values occurred within this period with each value reaching a plateau after approximately 40 min (ii) The inclusion of RQ and f_{exo} values after 40 min violated the assumption of homogeneity of covariance matrices (an important aspect of a mixed-design ANOVA). Mauchly's test of sphericity indicated data from the first 40 min satisfied assumptions of sphericity in most cases. We specifically note when data violated assumptions of sphericity below. Bonferroni correction was applied to *post hoc* comparisons among treatment means. Because ARCHCOL-025 died prior to its third experimental trial (sucrose), data from this bird were excluded in analyses that examine variation across all three treatment types. In analyses that examine differences between glucose and fructose treatments only, data from this bird are included.

In addition to possible differences in physiological flux capacities, variation in energy ingestion rate or energy turnover rate could conceivably have an effect on the estimated kinetics of incorporation of exogenous sugar into the pool of actively metabolized substrates. In particular, we suspected energy intake or expenditure rates over the first 40 min of the experimental period might have an effect on sugar incorporation kinetics because this was when $\delta^{13}C_{breath}$ values were expected to rise most dramatically (Welch *et al.* 2006; Welch & Suarez 2007). We had no a priori expectations regarding the relative influences of physiological vs. behavioural parameters on exogenous sugar oxidation kinetics. Thus, we adopted a multimodel inference approach as outlined by Burnham and Anderson (Burnham & Anderson 2002; Burnham, Anderson & Huyvaert 2011) to identify the most plausible model of the fractional rate of isotope incorporation into the pool of actively metabolized substrates. We fitted models only to fructose or glucose trials because we were most interested in understanding the relative kinetics of the use of each monosaccharide and because the double energy density of, and additional digestive steps needed to process, sucrose solutions introduced confounds to interpretation of the fitting exercise. Mixed effects models incorporating bird as a random factor in each case and, in additive combination, sugar type and the rate of energy ingestion over the first 40 min of the trial period were fitted to observed variation in the fractional rate of isotope incorporation into the pool of expired CO_2 (k_i) in the first 150 min of the experiment. Interaction parameters were not included due to diminished degrees of freedom. Models were fitted using the lme4 package (Bates, Maechler & Bolker 2012) in R v.2.15.3 (R Development Core Team 2011). Akaike's information criterion corrected for small sample size (AICc) was used as a measure of model fit and the probability that each model was the best fit was defined by its AIC weight (Burnham, Anderson & Huyvaert 2011). For these analysis, data from all hummingbirds ($n = 6$) were included as all were able to complete both glucose and fructose treatments.

SPSS (Armonk, New York, USA) version 17.0 software program was used to perform all statistical analyses in this study except for those noted directly above. All data are presented as mean ± SEM unless otherwise indicated. All data regarding amount of carbohydrate ingested or oxidized is expressed in joules, to account for the different energy densities of each solution, unless otherwise noted.

Results

RESPIRATORY QUOTIENT VALUES

Respiratory quotient (RQ) values displayed by ruby-throated hummingbirds during hovering flight rapidly increased when feeding on fructose, glucose and sucrose from *c*. 0·71 to 1·0 as birds transitioned from a fasted to a fed state (Fig. 1a). During the first feeding bout after a fast, RQ values were 0·77 ± 0·02 in fructose trials (n = 5), 0·76 ± 0·02 (n = 5) in glucose trials, and 0·75 ± 0·02 (n = 5) in sucrose trials, which indicated the hummingbirds were primarily oxidizing fatty acids to fuel flight when fasted (Fig. 1a). Within each treatment, RQ values increased significantly between 0 and 40 min ($F_{2,24}$ = 35·335, $P < 0·001$, $\eta_p^2 = 0·746$ with sphericity being met, $\chi^2(2 = 0·297$, $P = 0·862$, GLM-ANOVA). There was no significant interaction observed between RQ and treatment ($F_{4,24} = 1·253$, $P = 0·316$, $\eta_p^2 = 0·173$). Furthermore, RQ values did not differ significantly among treatments between 0 and 40 min ($F_{2,12} = 0·524$, $P = 0·605$, $\eta_p^2 = 0·080$). Between 50 and 150 min RQ values were 0·94 ± 0·02 (n = 5) for fructose, 0·94 ± 0·02 (n = 5) for glucose and 0·96 ± 0·03 (n = 5) for sucrose trials and did not vary significantly among treatments ($F_{2,8} = 0·570$, $P = 0·587$; RM-ANOVA), indicating that birds had switched to mainly oxidizing carbohydrates and continued doing so for the remainder of the experiment.

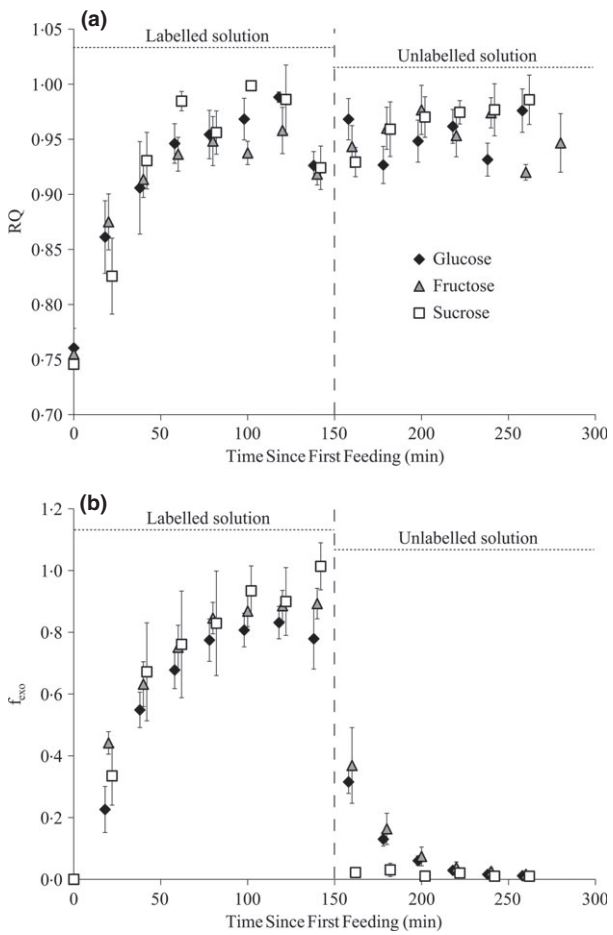

Fig. 1. Respiratory quotient (RQ; panel a) and proportion of expired CO_2 produced from the oxidation of isotopically-labelled solution given to the hummingbirds (f_{exo}; panel b) as a function of time since the first feeding following an overnight fasting period. Average data from all voluntary feedings from each individual within 20 min bins were averaged among individuals within treatment. Data are presented as these means (± SEM) and are offset slightly within each bin for visibility. The isotopically labelled solution was replaced with an equivalent unlabelled solution at the 150 min mark (dashed line) within each trial.

PROPORTION OF METABOLISM SUPPORTED BY EXOGENOUS SUGAR

During the period of availability of enriched solutions (first 150 min), the proportion of metabolism supported by exogenous 'enriched' sugar (f_{exo}) increased from *c*. 0% to near 100%, with values for some individuals reaching 100% in each treatment. For all three treatments, f_{exo} significantly increased for the first 40 min ($F_{2,24} = 70·734$, $P < 0·001$, $\eta_p^2 = 0·855$ with sphericity being met, $\chi^2(2) = 1·152$, $P = 0·562$, GLM-ANOVA). In addition, f_{exo} values did not vary significantly differently among treatments during this time period ($F_{2,12} = 1·148$, $P = 0·350$, $\eta_p^2 = 0·161$). The interaction of f_{exo} and treatment was found not to be significant, ($F_{4,24} = 0·737$, $P = 0·576$, $\eta_p^2 = 0·109$). On average, f_{exo} values from 50 to 150 min reached plateau values of 0·88 ± 0·01 (n = 5), 0·81 ± 0·04 (n = 5) and 0·92 ± 0·06 (n = 5) for fructose, glucose and sucrose, respectively (see Fig. 1b), and did not differ significantly among treatments ($F_{1·037,\ 4·150} = 0·424$, $P = 0·556$; RM-ANOVA, with a Greenhouse-Geisser correction).

CARBOHYDRATE OXIDATION KINETICS

The fractional rate of isotopic incorporation into the pool of expired CO_2 (k_i) did not vary significantly among individuals. k_i averaged 0·036 ± 0·004 (min^{-1}; range 0·024–0·048, n = 5, Table 2) for fructose, 0·023 ± 0·004 (min^{-1}; range 0·014–0·035, n = 5, Table 2) for glucose and 0·030 ± 0·007 (min^{-1}; range 0·008–0·051, n = 5, Table 2) for sucrose. No significant difference was observed for k_i between treatments ($F_{2,8} = 2·584$, $P = 0·136$; RM-ANOVA).

After 150 min since the first feeding, the enriched solutions were replaced with their respective unlabelled solutions. f_{exo} values decreased from *c*. 100% to 0% (Fig. 2b). The fractional rate of isotopic disappearance from the pool of expired CO_2 (k_d) averaged 0·054 ± 0·007 (min^{-1}; range 0·033–0·072, n = 5, Table 2) for fructose, 0·049 ± 0·006 (min^{-1}; range 0·028–0·061, n = 5, Table 2) for glucose and 0·041 ± 0·003 (min^{-1}; range 0·033–0·052, n = 5, Table 2) for sucrose. No significant difference was observed for k_d between treatments ($F_{2,8} = 1·045$, $P = 0·395$; RM-ANOVA). Within treatments, k_d was significantly higher than k_i for glucose ($t_{(4)} = 7·815$, $P = 0·001$), but not significantly different than k_i for fructose ($t_{(4)} = 2·123$, $P = 0·101$) and sucrose treatments ($t_{(4)} = 1·775$, $P = 0·151$).

Table 2. Kinetics of change in $\delta^{13}C_{breath}$ values for the first and second half of the experiment period when hummingbirds were offered fructose, glucose or sucrose solutions

	k_i (min^{-1}) 0–150 min			k_d (min^{-1}) 150–300 min		
ID	Fructose	Glucose	Sucrose	Fructose	Glucose	Sucrose
ARCHCOL-025*	0·028	0·024	N/A	0·062	0·069	N/A
ARCHCOL-017	0·035	0·026	0·008	0·062	0·056	0·035
ARCHCOL-033	0·024	0·017	0·021	0·047	0·051	0·042
ARCHCOL-008	0·048	0·014	0·036	0·058	0·028	0·052
ARCHCOL-013	0·043	0·035	0·051	0·033	0·061	0·044
ARCHCOL-026	0·032	0·021	0·033	0·072	0·048	0·033
Mean ± SEM	0·035 ± 0·004	0·023 ± 0·003	0·030 ± 0·007	0·056 ± 0·005	0·052 ± 0·006	0·041 ± 0·003

k_i, fractional rate of isotope incorporation into the pool of expired CO_2; k_d, fractional rate of isotope disappearance from the pool of expired CO_2.
*ARCHCOL-025 died prior to the sucrose treatment. Mean values shown include all six birds.

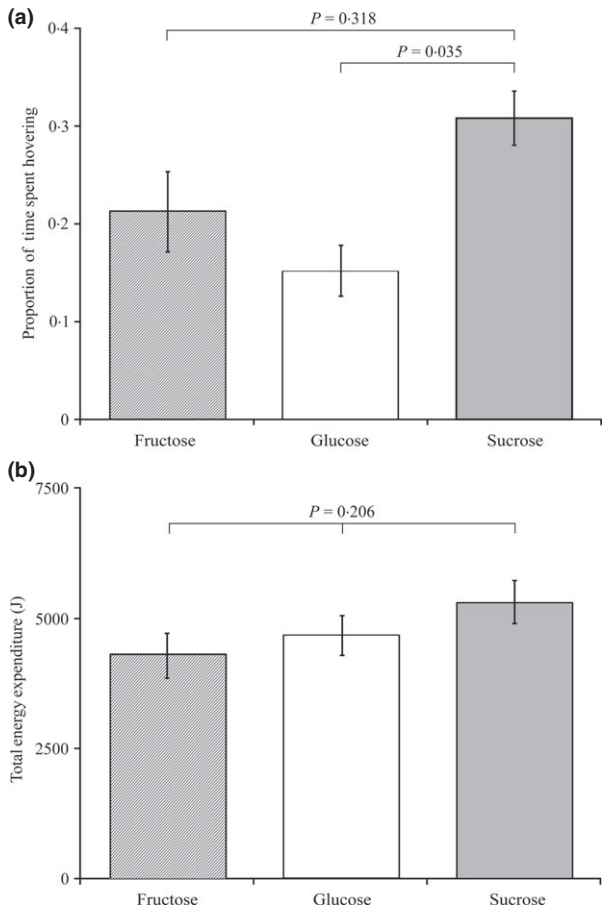

Fig. 2. Proportion of time spent hovering (a) and total energy expenditure, in joules, (b) over the first 150 min of the experiment for each treatment (sugar type). Data are presented as mean ± SEM.

ENERGY EXPENDITURE

Hummingbirds spent significantly different proportions of time hovering when different sugars were offered in the first 150 min ($F_{2,8}$ = 9·659, P = 0·007; RM-ANOVA). Hummingbirds offered sucrose spent a significantly greater proportion of their time hovering (0·31 ± 0·03; range: 0·21–0·37, n = 5; Fig. 2a) than when offered glucose (P = 0·035; 0·15 ± 0·03; range: 0·10–0·24, n = 5; Fig. 2a). The proportion of time spent hovering when offered sucrose was not significantly different from that which occurred when birds were offered fructose (P = 0·318; 0·21 ± 0·04, range: 0·11–0·34, n = 5; Fig. 2a) or. As a result, hummingbirds expended differing amounts of total energy in each treatment in the first 150 min (Fig. 2b). However, these differences were not significant among fructose (4325·37 ± 429·10 J, range: 3163·50–5415·92 J, n = 6), glucose (4691·77 ± 381·47 J, range: 3474·91–5559·05 J, n = 6) and sucrose (5325·86 ± 412·96 J, range: 4227·21–6380·22 J, n = 5) treatments ($F_{2,8}$ = 1·940, P = 0·206; RM-ANOVA, Fig. 2b).

ENERGY INTAKE AND AMOUNT OXIDIZED

In the first 150 min, the average amount (J) of carbohydrate ingested was similar among treatments ($F_{2,8}$ = 2·560, P = 0·138, RM-ANOVA). Birds ingested an average of 3307·74 ± 441·55 J (n = 5, Fig. 3a) of fructose, 2820·07 ± 439·45 J (n = 5, Fig. 3a) of glucose and 3771·60 ± 379·79 J (n = 5, Fig. 3a) of sucrose.

Hummingbirds oxidized similar amounts (J) of carbohydrates in the first 150 min of the experiment ($F_{2,8}$ = 0·172, P = 0·845, RM-ANOVA). On average, bird oxidized 2825·82 ± 322·80 J of fructose (n = 5, Fig. 3a), 2650·08 ± 221·17 J of glucose (n = 5, Fig. 3a) and 2545·53 ± 411·51 J of sucrose (n = 5, Fig. 3a). Hummingbirds oxidized similar percentages of the ingested energy in each treatment (fructose: 91·71 ± 14·34%, n = 5; glucose: 98·00 ± 7·05%, n = 5; sucrose: 67·56 ± 8·29%, n = 5; $F_{2,8}$ = 3·262, P = 0·092; RM-ANOVA; see Fig. 3b).

THE EFFECT OF BEHAVIOUR AND SUGAR TYPE ON OXIDATION KINETICS

The simplest model, which included only individual as a random factor, was judged to be the best fit as it had the

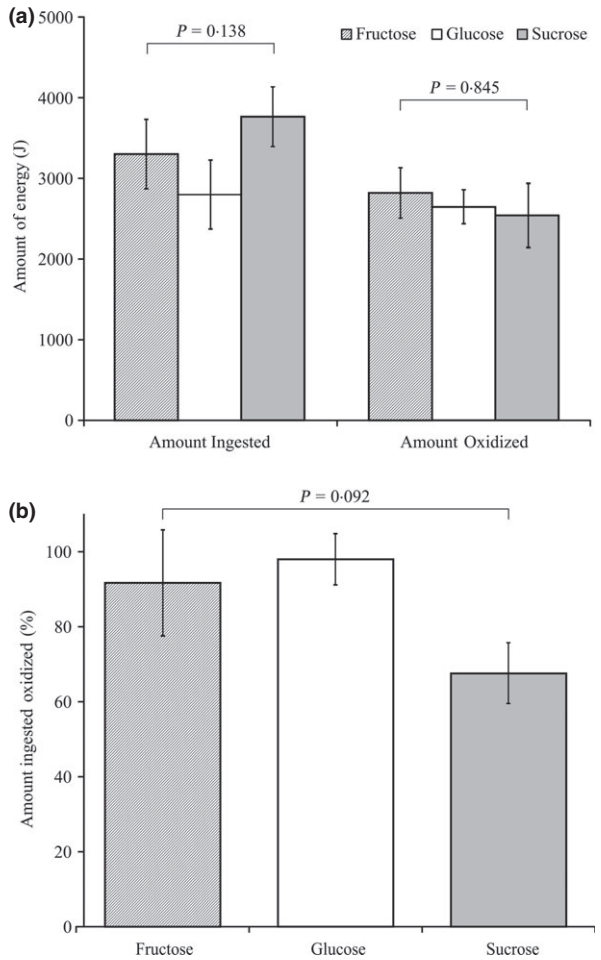

Fig. 3. The cumulative amount of energy ingested and oxidized, in joules, (a) and the percentage of ingested energy that was oxidized (b) during the first 150 min of the experimental period for each treatment (sugar type). This was the period of each trial when the isotopically labelled solution was available to the hummingbirds. Data are presented as mean ± SEM.

lowest corrected-Akaike's information criterion score (AICc = −60·3). The next best fit model (which included sugar type as a predictor) had an AICc score of −52·0. The Akaike weight (ω_i) for the simplest model was 0·981, indicating a >98% probability that this model was the best fit.

Discussion

RESPIRATORY QUOTIENTS AND CARBOHYDRATE OXIDATION KINETICS

Consistent with results obtained previously in rufous (Suarez *et al.* 1990; Welch, Altshuler & Suarez 2007), broad-tailed (Welch *et al.* 2006) and Anna's hummingbirds (Welch, Altshuler & Suarez 2007), RQ values displayed by ruby-throated hummingbirds during hovering flight rapidly increased for all three treatments from *c.* 0·71 to 1·0 as birds transitioned from a fasted to a fed state (Fig. 1a). This indicated that fatty acids were oxidized to provide most of the energy for hovering flight when birds were fasted. Subsequently, when given access to glucose, fructose or sucrose, hummingbirds increased their reliance on carbohydrates as fuel to support their hovering flight metabolism regardless of whether the available exogenous fuel was sucrose or either component monosaccharide.

During the first feeding following the fasting period, f_{exo} values were zero, and $\delta^{13}C_{breath}$ values were similar to the signature of the maintenance diet which indicated hovering metabolism was supported entirely by endogenous fatty acids, regardless of treatment (Fig. 1b). f_{exo} values increased over the first 40 min of feeding and plateaued thereafter. While average f_{exo} values for birds offered fructose or glucose were slightly <90% (0·88 ± 0·01 and 0·81 ± 0·04, respectively), some birds within each treatment did exhibit f_{exo} values approximately equal to 100%. Further, f_{exo} values did not vary significantly among treatments suggesting ruby-throated hummingbirds possess the capacity to fuel hovering metabolism entirely with newly ingested glucose or fructose, or with some mixture of the two component monsaccharides in the sucrose solution (see Fig. 1b).

The increase in f_{exo} values also mirrored the rise in RQ, which indicated the switch to reliance on carbohydrate oxidation to fuel hovering flight was a switch to exogenous fuel sources. RQ values remained near 1·0, despite f_{exo} values decreasing to zero when the 'enriched' solutions were replaced with 'unlabelled' solutions in the second half of the experiment. Similar results were obtained with rufous (Welch, Altshuler & Suarez 2007) and Anna's hummingbirds (Welch, Altshuler & Suarez 2007). The findings provide evidence that such physiological capacity of rapidly using only the most recently ingested sugars to fuel hovering flight is most likely common in all small hummingbirds.

Although sucrose solutions contained twice the energetic content per unit volume of the other solutions used in this study, the proportion of hovering metabolism supported by exogenous sucrose did not differ significantly between treatments. Research by Martinez del Rio (1990) and Schondube & Martínez del Rio (2003) showed that equicaloric sucrose and hexose mixtures of glucose and fructose are processed at equal rates in hummingbird intestine. The tracking of sugar flux from ingestion through to oxidation by flight muscle fibres also suggests no differences in the handling of either glucose or fructose at the level of the digestive tract, or elsewhere in the 'sugar oxidation cascade' (Suarez, Herrera & Welch 2011). The kinetics and extent of reliance on exogenous sugars were not even affected by the energy density of the nectar solution offered, as hummingbirds were able to support their hovering metabolism when feeding on fructose or glucose solutions just as rapidly as they were on a sucrose solution which contained double the energy density. Furthermore, the proportion of ingested energy oxidized was similar regardless of the identity of the sugars ingested.

In humans, it is thought skeletal muscles possess lower oxidative capacities for fructose relative to glucose and that fructose uptake is limited due to lower transport capacity (Kristiansen *et al.* 1997) that is reliant on a carrier-mediated transport system that does not involve GLUT4 or GLUT1 (Zierath *et al.* 1995). Human muscle cells lack the enzyme fructokinase, and because hexokinase, the entry point into glycolysis, has a markedly lower affinity for fructose than for glucose (Voet, Voet & Pratt 2008), it is very unlikely that fructose is directly metabolized in skeletal muscle at substantial rates. During exercise at 60% of $\dot{V}_{O_2}$max, the energy in ingested glucose and fructose solutions contribute 15 and 12%, respectively, to energy production in humans (Adopo *et al.* 1994). It is possible that, as in humans and other mammals, most of the absorbed fructose is rapidly taken up by the liver, converted into glucose, and re-released into circulation before it can be transported to, and oxidized by, active flight muscle fibers. However, the similar flux kinetics for sugars in glucose, fructose, and sucrose solutions suggests that hummingbird flight muscle fibres possess a much greater capacity for the direct oxidation of fructose than is seen in most other vertebrates.

If direct oxidation of fructose by flight muscle fibres does occur at much greater rates in hummingbirds than in other vertebrates there must be enhanced capacities for both the transport of fructose into muscle fibres as well as the use of fructose as a substrate, either directly or indirectly, by glycolysis. No data exist that directly quantify sugar uptake rates by hummingbird muscle fibres. However, it has been shown that hummingbird flight muscles demonstrate a high capacity for glucose phosphorylation to glucose-6-phosphate by the enzyme hexokinase (Suarez *et al.* 1990 and Suarez, Herrera & Welch 2011). Future studies are needed to determine whether hummingbird muscle fibre hexokinase possesses similar catalytic capacities for the phosphorylation of fructose as well, or if there is even a hexokinase isoform with high affinity for fructose (a fructokinase).

The fractional rates at which exogenous carbohydrates were incorporated into (k_i), or disappeared from (k_d), the pool of metabolically active subsrates were similar regardless of which sugars were ingested. The fractional rates of appearance and disappearance we observed in ruby-throated hummingbirds were similar to previously reported values in other small hummingbirds (Carleton, Bakken & Martínez del Rio 2006; Welch *et al.* 2006; Welch, Altshuler & Suarez 2007). Voigt & Speakman (2007) found nectar-feeding bats fuelled 82% and 95% of their metabolism with exogenous fructose and glucose respectively and suggested this was reflective of relative reliance on these fuels during energetically expensive flight as well. Interestingly, nectar bats were slower to incorporate newly ingested sugars into the pool of actively metabolized substrates (i.e. exhibited lower k_i values) when compared to ruby-throated, rufous, or Anna's hummingbirds, a difference possibly related to their higher body mass and lower mass-specific metabolic rate (Voigt & Speakman 2007; Welch & Suarez 2007; Welch, Herrera & Suarez 2008; Suarez, Herrera & Welch 2011).

BEHAVIOURAL ASPECTS OF THE STUDY

Interestingly, there appeared to be a correspondence between the amount of energy ingested and the amount of exogenous fuel energy oxidized in the first half of the experiment (see Fig. 3a). The amount of carbohydrates ingested (in joules) did not differ significantly among fructose, glucose and sucrose. Hummingbirds oxidized similar total caloric amounts of the fructose, glucose and sucrose they ingested. Consequently, the proportion of ingested carbohydrates that was oxidized (ratio of amount oxidized to amount ingested) also did not differ significantly among treatments. Our findings thus provide additional evidence that hummingbirds are capable of acute control over net energy gain regardless of nectar energy density or sugar composition (Carpenter, Paton & Hixon 1983; Gass, Romich & Suarez 1999).

Previous research has shown that hummingbirds feed more often and spend more time flying if less food energy is available per visit (Wolf & Hainsworth 1977; Gass, Romich & Suarez 1999). However, in this study, hummingbirds spent a significantly greater proportion of their time hovering when offered sucrose, compared to when fructose or glucose were available, despite the fact that the sucrose solution had twice the energetic density of glucose and fructose solutions. There were no significant differences in the total amount of energy expenditure and amount of energy ingested between the three treatments even though hummingbirds spent a greater proportion of their time hovering for sucrose than fructose or glucose. Differences in both perching and hovering metabolic rates among individuals and across trials led to calculation of less variation in energy expenditure among treatment types than is suggested by time budgets.

IS INDIVIDUAL VARIATION IN SUGAR OXIDATION KINETICS BEHAVIOUR OR PHYSIOLOGY MEDIATED?

The multimodel inference approach indicated that the simplest model (that which included only individual as a random factor) best predicted variation in k_i. ΔAICc values (difference in AICc score between a given model and that with the lowest AICc score) for all other models was > 8, indicating their plausibility was low (Burnham, Anderson & Huyvaert 2011). In addition, the probability that the simplest model was the best fit to the data was >98%, as indicated by relative Akaike weights (ω_i). Taken collectively, this strongly suggests that there is no physiologically based significant difference in the kinetics of incorporation of either fructose or glucose into the pool of actively metabolized substrates. Interestingly, while log-likelihood ratio scores indicated that inclusion of sugar type as a predictor improved the fit of the model to observed data

($\chi^2 = 6{\cdot}08$, $P = 0{\cdot}01$), exogenous fructose was, if anything, incorporated into the pool of actively metabolized substrates more rapidly than glucose, a pattern which is opposite that which would be predicted based on an understanding of sugar uptake and oxidation kinetics in most mammalian muscles (Delarue *et al*. 1993; Zierath *et al*. 1995; Kristiansen *et al*. 1997). While there was also evidence of a positive relationship between the rate of energy ingestion over the first 40 min of the trial and the fractional rate of incorporation of exogenous sugars into the pool of actively metabolized substrates, the plausibility of a model which incorporated this behavioural predictor was found to be quite low. Incorporation kinetics were largely similar given the range of ingestion rates observed, suggesting that each individual ingested nectar at a rate sufficient to permit rapid and complete switching to reliance on exogenous fuels.

EVOLUTIONARY AND ECOLOGICAL IMPLICATIONS

The ability of fasted hummingbirds to initially rely predominantly on fatty acids to fuel hovering flight is one they have in common with most other birds. Fatty acids are a principle fuel source during long distance migratory flights in both hummingbirds (Carpenter *et al*., 1993) and other species (Jenni & Jenni-Eiermann 1998; Guglielmo 2010). This specialization on fatty acids as a fuel source is hypothesized to be advantageous because it is the most energetically dense form of fuel storage (Jenni & Jenni-Eiermann 1998; Guglielmo 2010). The mass-saving advantages of deposition of energetically dense fat, as opposed to glycogen, are clear for small-bodied, hovering hummingbirds more so than for any other, larger, avian species. Thus, it is not surprising that hummingbirds can fuel flight exclusively with fat not only during long-distance migratory flights, but also during shorter fasting periods and prior to commencing foraging in the morning (Suarez *et al*. 1990).

Migrating birds also appear to rely to a variable extent on oxidation of amino acid oxidation to fuel flight (Jenni & Jenni-Eiermann 1998; Guglielmo 2010; Gerson & Guglielmo 2011), and this includes hummingbirds (Carpenter *et al*., 1993). However, the minimal daily nitrogen requirements of hummingbirds (López-Calleja, Fernández & Bozinovic, 2003) and the fact that they display RQ values near 0·7 following an overnight fast (Suarez *et al*. 1990; Welch & Suarez 2007; Welch, Herrera & Suarez 2008; this study) suggest that oxidation of amino acids play no substantial part in the fueling of day-to-day activities, including hovering flight.

In general, non-nectarivorous birds show a predisposition towards oxidation of fatty acids to fuel even nonmigratory flight. Most studies employing respirometry on birds flying in wind tunnels have reported respiratory quotient values of between 0·7 and 0·8, indicative of a reliance primarily on fatty acid oxidation (e.g. Tucker 1968, 1972; Torre-Bueno & Larochelle 1978; Hudson & Bernstein 1983; Ward *et al*. 2001; Bundle, Hansen & Dial 2007). There are fewer studies employing isotopic tracking to uncover the source of oxidative fuels in birds, particularly during flight. However, in one such study, Hatch, Pinshow & Speakman (2002) demonstrate that pigeons fasted as little as two hours displayed breath isotopic signatures immediately following flight which suggested that fatty acid oxidation accounted for the majority of oxidized fuels. Birds flown immediately following feeding displayed breath stable isotopic signatures at rest which suggested a roughly equal reliance on carbohydrates and fatty acids to fuel metabolism (Hatch, Pinshow & Speakman 2002). Because samples were obtained from resting/recovering but not currently flying birds it is not certain that carbohydrate oxidation contributes significantly to flight metabolism even in this study.

Hummingbirds appear unique among the birds thus far studied in their ability to rely exclusively on either glucose or fructose to fuel flight. We are unaware of any studies which examine the relative use of fructose as a fuel during flight in any other avian taxa. Because most birds seem not to rely on carbohydrates to fuel flight in general we suspect their ability to rely on fructose specifically is also limited.

The unusual capacity to rapidly and completely rely on newly ingested sugar to fuel foraging behaviour is undoubtedly related to the specialized dietary ecology of hummingbirds. Almost all birds for which fuel use during flight have been investigated are granivorous or insectivorous/carnivorous. Even generalist species (e.g. pigeon) do not typically ingest large quantities of simple sugars. None possesses a diet especially rich in fructose. However, little is known about patterns of fuel use during flight in avian frugivores and other nectarivores. Such species, like hummingbirds, take in a diet rich in simple sugars and, notably, fructose. The fuel use strategy of foraging nectar bats has, like their dietary ecology, converged with that of hummingbirds (Voigt & Speakman 2007; Welch, Herrera & Suarez 2008; Suarez, Herrera & Welch 2011). It thus seems possible that fuel use strategies during flight in avian frugivores and nectarivores may be quite similar to that of hummingbirds, including an ability to rely directly on ingested fructose.

The mean residence time of a carbon molecule within the pool of actively metabolized substrates can be determined by calculating the inverse of k. Based on k_d values, which are calculated during the period when hummingbirds are steadily relying on hexoses to fuel behaviour, recently ingested carbon atom remained in the pool of actively metabolized substrates for roughly 20 min on average (fructose: $19{\cdot}2 \pm 2{\cdot}4$ min; glucose: $20{\cdot}8 \pm 3{\cdot}1$ min; sucrose: $24{\cdot}9 \pm 2{\cdot}0$ min; Table 2). This number is striking given that the feeding frequency was roughly set to once every 15 min (based on when the feeder was made accessible to the bird). Interestingly, data from rufous and Anna's hummingbirds examined in a 2007 study by Welch and Suarez show a similar congruence between carbon

atom turnover and foraging frequency. In this study, carbon atoms derived from ingested sucrose remained in the pool of actively metabolized substrates for an average of $9{\cdot}7 \pm 1{\cdot}4$ and $17{\cdot}6 \pm 0{\cdot}6$ min in rufous and Anna's hummingbirds, respectively (Welch & Suarez 2007). In this study, feeding bouts occurred, on average, once every $10{\cdot}0 \pm 1{\cdot}8$ and $9{\cdot}4 \pm 1{\cdot}3$ min by rufous and Anna's hummingbirds, respectively (Welch & Suarez 2007). The correlation of carbon atom residence time and foraging bout frequency suggests a behavioural strategy for maximizing net energetic yield. Hummingbirds appear to behave as 'carbohydrate maximizers' during foraging (Suarez *et al*. 1990). By limiting delays between foraging bouts to periods less than or equal to the average residence time of a carbon atom in the pool of actively metabolized substrates, hummingbirds ensure that the newly ingested hexoses remain sufficiently available to active tissues so as to ensure complete reliance on carbohydrates and sparing of lipid reserves. This strategy ensures both that the birds do not pay a 16% energetic tax associated with oxidation of lipids synthesized from ingested sugars and that they thus achieve optimal rates of lipid deposition (Suarez *et al*. 1990; Suarez, Herrera & Welch 2011), the latter most apparent in the rufous hummingbirds which were, at the time of the experiment, in a pre-migratory phase.

Remarkably, this study shows that the benefits of this fuel use strategy are likely realized regardless of which component hexose is considered. Because ruby-throated hummingbirds apparently show just as much capacity to rely on newly ingested fructose as they do glucose, the advantages of using newly ingested sugar to fuel foraging are realized across the entirety of each ingested nectar meal. This suggests that the remarkable suite of functional enhancements that enable the rapid uptake, distribution and oxidation of newly ingested sugar termed the 'sugar oxidation cascade' (Suarez, Herrera & Welch 2011) may also involve fundamental differences in the handling of fructose at the level of active muscle tissue.

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