

RESEARCH ARTICLE

Metabolic partitioning of sucrose and seasonal changes in fat turnover rate in ruby-throated hummingbirds (*Archilochus colubris*)

Morag F. Dick^{1,*}, Antonio Alcantara-Tangonan¹, Yazan Shamli Oghli¹ and Kenneth C. Welch^{1,2}

ABSTRACT

Hummingbirds fuel their high energy needs with the fructose and glucose in their nectar diets. These sugars are used both to fuel immediate energy needs and to build fat stores to fuel future fasting periods. Fasting hummingbirds can deplete energy stores in only hours and need to be continuously replacing these stores while feeding and foraging. Whether and how hummingbirds partition dietary fructose and glucose towards immediate oxidation versus fat storage is unknown. Using a chronic stable isotope tracer methodology, we examined whether glucose or fructose is preferentially used for *de novo* lipogenesis in ruby-throated hummingbirds (*Archilochus colubris*). Potential seasonal changes were correlated with variation in the overall daily energy expenditure. We fed ruby-throated hummingbirds sucrose-based diets enriched with ¹³C on either the glucose or the fructose portion of the disaccharide for 5 days. Isotopic incorporation into fat stores was measured via the breath ¹³C signature while fasting (oxidizing fat) during the winter and summer seasons. We found greater isotopic enrichment of fat stores when glucose was labelled compared with fructose, suggesting preference for glucose as a substrate for fatty acid synthesis. We also found a seasonal effect on fat turnover rate. Faster turnover rates occurred during the summer months, when birds maintained lower body mass, fat stores and exhibited higher daily nectar intake compared with winter. This demonstrates that fat turnover rate can substantially vary with changing energy expenditure and body composition; however, the partitioning of sucrose towards *de novo* fatty acid synthesis remains constant.

KEY WORDS: Hummingbirds, Sugar metabolism, Stable isotope analysis, Lipogenesis, Fat turnover

INTRODUCTION

Animals need to manage both current and future energy needs when food is not available as part of balancing their energy budgets. Hummingbirds, like other small euthermic animals, have high mass-specific metabolic rates, but additionally must manage the costs of energy-demanding flight (Suarez, 1992), making their daily energy demand high. Seasonal changes in energy requirements and body mass, such as those associated with moulting, breeding and migration (Hiebert, 1991; Hou and Welch, 2016), may also

influence how hummingbirds use ingested nectar for immediate versus long-term needs. Each meal consumed contributes not only to fueling immediate energy expenditure, but also to the building of energy reserves to fuel behavior between meals and longer periods of fasting such as overnight or during migratory flights.


Hummingbirds consume a predominately nectar-based diet. As such, hummingbirds fuel their lifestyles primarily with the disaccharide sucrose, and its monomers fructose and glucose found in the nectar (Baker and Baker, 1990). Thriving on a simple carbohydrate diet means that these sugars are the oxidative substrate used both for current needs and as the primary substrates for building fat stores via *de novo* lipogenesis. Ingested nectar is quickly assimilated and can be used to directly fuel almost all of their immediate energy needs in less than 15 min after consumption (Carleton et al., 2006; Chen and Welch, 2014; Welch and Suarez, 2007; Groom et al., 2019). Interestingly, hummingbirds can fuel their metabolism, including energetically demanding hovering flight, with either fructose or glucose (Chen and Welch, 2014). In addition to their ability to quickly assimilate and oxidize sugar, hummingbirds also use ingested sugar for *de novo* lipogenesis (Eberts et al., 2019). The liver is the principal site of lipogenesis in birds (Blem, 1976) and the hummingbird liver has one of the highest capacities for *de novo* lipogenesis seen among vertebrates (Suarez et al., 1988). The capacity to rapidly use recently ingested nectar to fuel flight is the result of a suite of adaptations that enable quick absorption from the intestine, circulation and tissue level cellular uptake and enzymatic capacity for both glucose and fructose, termed the sugar oxidation cascade (Welch et al., 2018). These adaptations also likely enable the rapid capacity for lipogenesis.

Whether and how animals metabolically partition dietary sucrose, specifically its glucose and fructose portions, towards immediate versus long-term needs is unknown in nectar specialists and poorly characterized in model species. Substrate preferences for fatty acid synthesis in rodent models suggests that when fructose intake is low, fructose is the preferred substrate for fatty acid synthesis, but when fructose intake is high, glucose is the preferred substrate (Carmona and Freedland, 1989; Chevalier et al., 1972; Romsos and Leveille, 1974). Preferential partitioning of glucose or fructose towards lipogenesis may depend on how it is used to fuel immediate metabolism. In healthy humans, the fructose portion of sucrose molecule is oxidized sooner and its oxidation peaks before that of glucose (Daly et al., 2000). Additionally, under normal fructose consumption, a large proportion of ingested fructose may be metabolized to glucose, lactate or other metabolites (Jang et al., 2018), which could also contribute to metabolic partitioning. This suggests that if fructose is rapidly metabolized or oxidized, glucose could be diverted towards lipogenesis, where it may be the preferred substrate.

An important differentiating factor is that hummingbirds, unlike rodents and humans, have evolved to consume a diet high in both

¹Department of Biological Sciences, University of Toronto Scarborough, 1265 Military Trail, Toronto, Ontario, M1C 1A4, Canada. ²Department of Cell & Systems Biology, University of Toronto, 27 King's College Circle, Toronto, ON, M5S 3G5, Canada.

*Author for correspondence (morag.dick@utoronto.ca)

 M.F.D., 0000-0002-7408-9656; K.C.W., 0000-0002-3283-6510

glucose and fructose. Thus, hummingbirds may have adaptations to metabolic pathways that distinguish how they use dietary sugars compared with typical models. We proposed that hummingbirds would preferentially use fructose for lipogenesis, sparing glucose to fuel hovering flight (Welch et al., 2018). This prediction was based on the greater capacity of flight muscle enzymes to phosphorylate glucose, a rate-limiting step in glycolysis, and the presence of fructose-specific catalytic enzymes in the liver but not flight muscles (Myrka and Welch, 2018). Additionally, along with a high capacity for mediated glucose uptake (Karasov et al., 1986), hummingbirds, similar to other small birds, are reliant on paracellular transport to achieve high sugar absorption rates (McWhorter et al., 2006). This suggests that enterocyte contribution to sugar metabolism is less significant than in traditional mammalian models.

Limited available evidence indicates that in comparison, small animals turn over fat stores more rapidly. This is in part because higher tissue turnover rates are predicted for small animals with high mass-specific metabolic rates (Brown, 2000; Voigt et al., 2003). This causes the relatively small fat stores to have high rates of deposition and use, and thus rapid turnover (Carleton et al., 2006). For example, broad-tailed hummingbirds (*Selasphorus platycercus*) turn over 50% of carbon in fat stores (termed t_{50}) in approximately 25 h (Carleton et al., 2006). Small mammals also exhibit rapid fat turnover rates, with a t_{50} of 21 h in Peters' tent-making bat (*Urogerma bilobatum*; O'Mara et al., 2017), and the exceptionally fast t_{50} of less than 5 h in the common shrew (*Sorex araneus*; Keicher et al., 2017). Turnover rates decrease when examining slightly larger animals, such as gerbils (*Merion unguierlatus*), that have a t_{50} of ~15.6 days (Tieszen et al., 1983). These studies also illuminate how life stage, energy balance and body mass can influence turnover rate. For instance, in broad-tailed hummingbirds, fat turnover rates were faster when in a positive energy balance (i.e. increasing fat stores) compared with when in a negative energy balance (Carleton et al., 2006). In common shrews, life stage, in combination with changes in body mass, influences fat turnover rate. Larger juveniles have slower turnover rates than smaller sub-adult individuals (Keicher et al., 2017). Furthermore, the rate of carbon incorporation generally scales negatively with body mass in birds (Carleton and Martinez del Rio, 2005). Thus, changes in body mass, fat reserves, energy expenditure and life history traits may influence the rate of fat turnover in animals (Brown, 2000; Keicher et al., 2017). Seasonal and environmental differences contributed to a 3-fold difference in daily energy expenditure in broad-tailed hummingbirds (Shankar et al., 2019). This suggests that seasonal changes in fat turnover rates could occur in hummingbirds in coordination with seasonal changes in body mass and daily energy expenditure.

Fat turnover rates have been studied by assessing variation in the stable carbon isotopic breath signatures of animals when lipid stores have been isotopically labeled (Carleton et al., 2006; Keicher et al., 2017; O'Mara et al., 2017). This is achieved by switching animals from a diet with one carbon stable isotope signature to a diet with another stable isotope signature. The incorporation of the new dietary carbons into fat stores is then measured by monitoring the breath stable isotope signature of fasting animals (McCue and Welch, 2016). This approach could also be used to test for metabolic partitioning of dietary components by selectively enriching different dietary nutrients with ^{13}C and assessing their incorporation into fat stores. We used this approach to test for the partitioning of dietary sucrose for lipogenesis and assessed seasonal changes in fat turnover rate. We chronically fed ruby-throated hummingbirds diets enriched with ^{13}C on either the glucose or

fructose portion of sucrose and tracked its incorporation into fat stores via fasting breath carbon stable isotope signatures. Additionally, we assessed metabolic partitioning over two seasons to determine whether fat turnover rate remains constant despite changing energy use.

MATERIALS AND METHODS

We report $\delta^{13}\text{C}$ on a per mil (‰) basis relative to the standard international carbon reference, Vienna Pee Dee Belemnite (VPDB, $^{13}\text{C}/^{12}\text{C}=0.01123$):

$$\delta^{13}\text{C} = \left[\frac{\left(\frac{^{13}\text{C}}{^{12}\text{C}} \right)_{\text{sample}}}{\left(\frac{^{13}\text{C}}{^{12}\text{C}} \right)_{\text{standard}}} - 1 \right] \times 10^3. \quad (1)$$

Animals

Male ruby-throated hummingbirds were caught during the summer of 2017 for the winter trials and during the summer of 2018 for the summer trials using modified box traps. The birds were housed individually in cages (91×51×54 cm) at the University of Toronto Scarborough campus vivarium. Animals were fed a 3:1 Nektar-Plus (Nekton, Tarpon Springs, FL, USA):sucrose diet (18% w/v) *ad libitum*, using 10 ml amber syringes that were replaced daily. The birds were kept on a photoperiod of 13 h:1 h light:dark during the summer, and 11 h:13 h light:dark during the winter. All protocols and animal collection procedures were approved by and in accordance with the requirements of the University of Toronto Animal Care Committee and the Canadian Wildlife Service.

Study design

We randomly blocked the birds into three groups of two birds for winter trials and two groups of three birds for summer trials. We employed a crossover design, with each individual being subjected to each treatment. We tested for the preferential incorporation of sugars by randomly allocating the birds to a diet with sucrose enriched with ^{13}C on the glucose or fructose portion for 5 days. The birds had a 14-day washout period between the glucose and fructose trials to allow their fasting $\delta^{13}\text{C}$ signatures to return to the baseline values reflecting their maintenance nectar diet.

To assess the incorporation of the enriched sugars into the fat stores, we measured the $\delta^{13}\text{CO}_2$ signature of the birds' breath each morning at the beginning of the light period. This time was chosen to ensure the birds were in a fasted condition and to minimize the contribution of *de novo* lipid stores built during the day (McCue and Welch, 2016; Fisher and Bartlett, 1957). On day 1, the birds' baseline $\delta^{13}\text{CO}_2$ breath signatures, rates of oxygen (\dot{V}_{O_2}) and carbon dioxide production (\dot{V}_{CO_2}), and respiratory exchange ratio ($\text{RER}=\dot{V}_{\text{CO}_2}/\dot{V}_{\text{O}_2}$) were measured or calculated. After this, the birds started the experimental ^{13}C -enriched diet.

^{13}C nectar enrichment

To test for metabolic partitioning of dietary glucose or fructose, we labeled the maintenance nectar diet with sucrose enriched with ^{13}C on all six carbons of either the glucose [D -sucrose (glucose- $^{13}\text{C}_6$, 98%), Cambridge Isotope Laboratories, Tewksbury, MA, USA] or fructose [D -sucrose (fructose- $^{13}\text{C}_6$, 98%), Cambridge Isotope Laboratories] portion of the sucrose molecule. The carbon isotopic signatures of the whole maintenance and labeled diets are shown in Table 1.

Table 1. Stable carbon isotope signatures ($\delta^{13}\text{C}$) of maintenance diet and glucose- and fructose-enriched diets

	$\delta^{13}\text{C}$ (‰ VPDB)
Maintenance	-17.60±0.15
Winter	
Glucose labeled	5.81±0.66
Fructose labeled	3.49±0.78
Summer	
Glucose labeled	5.32±0.37
Fructose labeled	2.46±0.32

Values are means±s.e.m., $n=4$ per diet. VPDB, Vienna Pee Dee Belemnite ^{13}C standard.

Respirometry and carbon stable isotope analysis

Birds were placed in 500 ml respirometry chambers fitted with perches. Flow rate into the chambers was maintained at 300 ml min^{-1} and the birds were provided with dry CO_2 -free air (Ultra Zero Air, Praxair, Canada) with the flow rate controlled by a multichannel flow controller (Flowbar-4 Mass Flow Meter System, Sable Systems International, Las Vegas, NV, USA). Air exiting the chambers flowed into a multiplexer (RM-8 Flow Multiplexer, Sable Systems International) and the air exiting the multiplexer was subsampled for respirometry at 100 ml min^{-1} , and for carbon stable isotope analysis at 35 ml min^{-1} . Subsampled air for respirometry was sequentially pulled through a water vapor pressure analyzer, carbon dioxide analyzer, drying column (Drierite, W. A. Hammond Drierite, Xenia, OH, USA) and oxygen analyzer using the incorporated subsampling pump (Turbofox-5, Sable Systems International). It was recorded using Expedata software (v. 1.8.4, Sable Systems International). Analyzers were calibrated regularly using well-mixed dry ambient air for the oxygen analyzer, 0% and 0.25% CO_2 reference gas for the carbon dioxide analyzer, and dry air and a dew point generator for the water vapor analyzer. We used the raw gas measurement to calculate \dot{V}_{O_2} and \dot{V}_{CO_2} by first drift- and lag-correcting the data and then applying standard equations (Lighton, 2018). Three-minute averages were taken from the last measurement of the hour, when the metabolic rate and $\delta^{13}\text{C}$ values were the most stable, to determine RER and $\delta^{13}\text{C}_{\text{breath}}$ signatures. The RER was measured to validate that the hummingbirds were in a fasted condition and that CO_2 exhaled was predominantly derived from lipid oxidation (Kleiber, 1975; Lighton, 2008).

Breath and nectar samples were analyzed for their $\delta^{13}\text{C}$ signatures via cavity ring-down spectroscopy using the Picarro G2201-i Analyzer (Picarro, Inc., Santa Clara, CA, USA). Breath samples were continuously measured from the excurrent multiplexer air. Liquid diet samples were analyzed with the companion Combustion Module and Caddy (Picarro). The nectar samples were prepared by placing 1 to 1.5 mg of celite (Costech Analytical Technologies, Inc., Valencia, CA, USA) and 5 μl of nectar into a 4×6 mm tin capsule (Costech Analytical Technologies), which was pinched closed. For every 10 samples, an acetanilide standard (Costech Analytical Technologies) was run and the system calibration was checked at the start of each season.

To standardize the $\delta^{13}\text{C}$ comparisons between the diets, we express the proportion of CO_2 in the breath derived from the experimental diets as f_{exo} (Welch and Suarez, 2007):

$$f_{\text{exo}} = \frac{\delta^{13}\text{C}_{\text{breath}} - \delta^{13}\text{C}_{\text{maintenance}}}{\delta^{13}\text{C}_{\text{acute}} - \delta^{13}\text{C}_{\text{maintenance}}}, \quad (2)$$

where $\delta^{13}\text{C}_{\text{breath}}$ is equivalent to $\delta^{13}\text{C}(t)$ at time t , $\delta^{13}\text{C}_{\text{maintenance}}$ is the breath delta signature at time 0 and $\delta^{13}\text{C}_{\text{acute}}$ is the isotopic signature of the trial diet. We estimated the turnover of carbon in the

adipose tissue using a one-pool exponential model to estimate the fractional incorporation of labeled sugars into the fat stores following Chen and Welch (2014):

$$f_{\text{exo}}(t) = f_{\text{exo}\infty} + (f_{\text{exo}t=0} - f_{\text{exo}\infty})e^{-kt}, \quad (3)$$

where $f_{\text{exo}\infty}$ is the asymptote, k is the rate of carbon incorporation per hour and t is time in hours. We estimated the t_{50} , the time it takes for 50% of the carbons in the fat pool to turn over, as follows (Keicher et al., 2017):

$$t_{50} = -\frac{\ln(0.5)}{k}. \quad (4)$$

Statistical analysis

Statistical analysis was performed using R (version 3.5.2). Daily body mass and nectar intake were analyzed using mixed-effect models and ANOVA (lmer function of the lme4 package; Bates et al., 2015) using hour, diet and season as fixed effects, and bird ID and block as random effects. The breath $\delta^{13}\text{C}$ signatures were used to fit individual curves to Eqn 3 above using nonlinear least-squares estimates (nlstools; Baty et al., 2015) of the k and $f_{\text{exo}\infty}$ parameters and setting the initial breath $t=0$ to 0. To analyze for differences in model parameter estimates, we used mixed-effect models using hour and diet as fixed effects and block and bird ID as random effects. There was no effect of diet order on the any measured variable and it was dropped from the models ($P>0.2$).

To assess the relationship of k and t_{50} to variation in body mass and nectar intake, we first used repeated-measures correlations (rmcorr; Bakdash and Marusich, 2017), which test for within-individual correlations. No significant correlation was found. We then averaged the values for diet trials and tested for significant relationships using Pearson's correlation coefficient. Finally, we generated a multiple regression model of k using nectar intake and body mass as factors. Statistical significance was set at $\alpha=0.05$, and values are reported as means±s.e.m.

RESULTS

Body mass and nectar intake

We found a significant interaction between season and hour for body mass ($F_{5,110}=27$, $P<0.0001$; Fig. 1). Body mass of the birds significantly differed at the start of the study, with the summer birds weighing 47% less than the winter birds. This difference was maintained throughout all the trials. Furthermore, in the winter trials, the birds lost mass over the course of 5-day trials, losing on average 0.3 g.

Hummingbirds naturally vary nectar intake from day to day. As such, we analyzed average daily nectar intake over the course of each trial. We found that average daily nectar intake differed seasonally and was 73% higher in the summer ($F_{1,10}=74.25$, $P<0.0001$; Fig. 2). It did not statistically differ between diet treatments ($F_{1,10}=0.0014$, $P=0.97$), nor was the interaction between the two factors statistically significant ($F_{1,10}=0.31$, $P=0.59$).

Initial $\delta^{13}\text{C}$ values and RER

The initial $\delta^{13}\text{C}$ breath signatures did not differ between the winter and summer seasons ($F_{1,10}=3.55$, $P=0.09$), or between the sugar trials ($F_{1,10}=0.79$, $P=0.39$). Furthermore, the interaction of the two factors was not statistically significant ($F_{1,10}=0.80$, $P=0.41$). Mean daily RER was 0.70 ± 0.01 , indicating that the birds were in a fasted condition and fueling their metabolism primarily with fat during the morning breath analysis (Kleiber, 1975; Lighton, 2008).

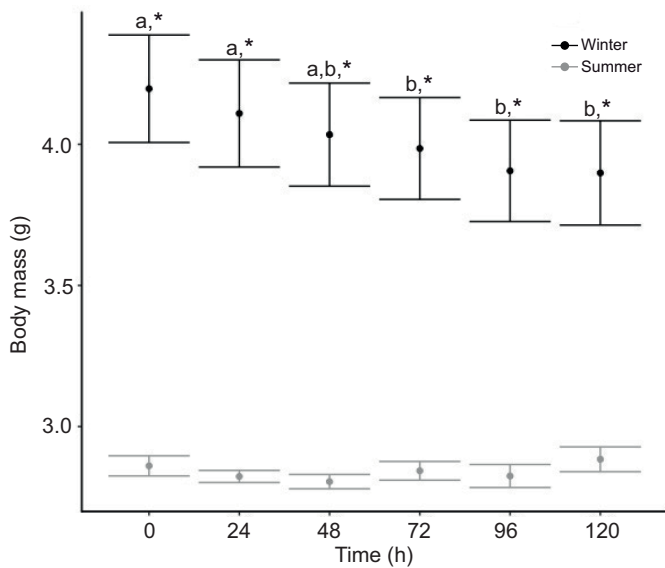


Fig. 1. Average daily body mass of ruby-throated hummingbirds (*Archilochus colubris*) during the 5-day trials during the winter and summer seasons. Values represent seasonal means \pm s.e.m. * indicates a significant seasonal difference ($P < 0.05$). Values that do not share a common letter differ within the winter season.

Incorporation rate of sucrose with ^{13}C -enriched glucose and ^{13}C -enriched fructose

Over the course of 5-day trials, the proportion of CO_2 from the experimental diets, f_{exo} , increased as the birds incorporated the carbon from the nectar into their fat stores (Fig. 3A). Whether the diets were enriched with the ^{13}C glucose or fructose significantly influenced the asymptotic isotopic values of exhaled CO_2 , $f_{\text{exo},\infty}$ ($F_{1,10}=45.08$, $P < 0.0001$), but did not affect the carbon incorporation rate, k ($F_{1,20}=0.27$, $P=0.61$; Fig. 3B). The ^{13}C glucose–sucrose enriched trials had significantly higher $f_{\text{exo},\infty}$ estimates than the ^{13}C fructose–sucrose trials (1.00 ± 0.05 for

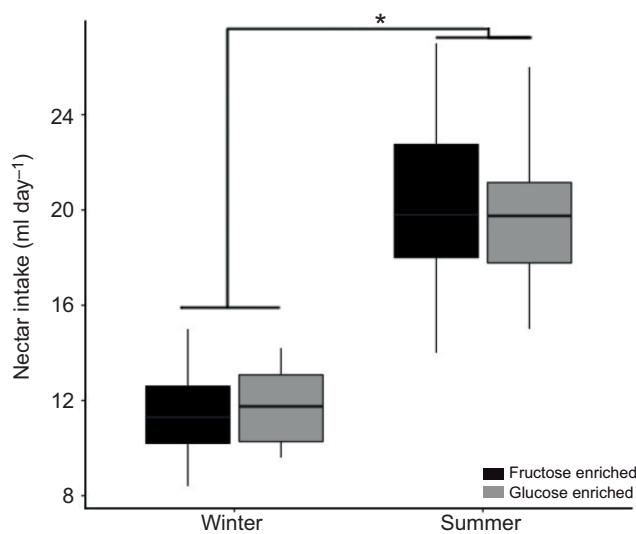


Fig. 2. Daily nectar intake during the 120 h trials for *A. colubris* fed nectar diets with sucrose molecules enriched with ^{13}C fructose (black) or ^{13}C glucose (grey) during the winter or summer seasons. The limits of the boxes represent the 25th and 75th percentiles, the bar the median, and the whiskers represent the maximum value, or 1.5 times the interquartile range. Values are $n=6$ per season.

glucose versus 0.62 ± 0.03 for fructose). Additionally, we found a significant effect of season for both $f_{\text{exo},\infty}$ and k in the model estimates of fat turnover rate. The $f_{\text{exo},\infty}$ was overall significantly higher in summer than winter (0.88 ± 0.06 in summer versus 0.75 ± 0.07 in winter; $F_{1,10}=5.24$, $P=0.04$; Fig. 3C). Furthermore, k , the parameter reflecting the rate of isotope incorporation, was significantly greater during the summer trial ($0.0282 \pm 0.0027 \text{ h}^{-1}$ in summer versus $0.00928 \pm 0.001 \text{ h}^{-1}$ in winter; $F_{1,20}=39.23$, $P < 0.001$).

Fat turnover rate relationship with behavior

The time for 50% of the carbons to turn over, t_{50} , differed significantly between seasons ($F_{1,10}=22.15$, $P < 0.001$; Fig. 4), but did not differ between trials ($F_{1,10}=0.65$, $P=0.44$) and there was no significant interaction between the two factors ($F_{1,10}=0.33$, $P=0.57$). The t_{50} was 3.3 times longer in the winter ($97.4 \pm 9.5 \text{ h}$) than in the summer ($26.7 \pm 2.1 \text{ h}$). To examine factors that may influence fat turnover rate, we correlated k and t_{50} with body mass and nectar intake. Initially, we used repeated-measures correlations and did not detect any significant relationship between t_{50} with body mass ($r=0.18$, $P=0.55$) or nectar intake ($r=0.10$, $P=0.74$). Because there was no among-individual variation in the trials, we averaged the glucose and fructose trials for each bird for the correlation analysis. Using the average values, we found a significant strong simple correlation for body mass with k ($r=0.86$, $P < 0.0001$) and t_{50} ($r=0.93$, $P < 0.0001$; Fig. 5A), and a significant simple correlation for nectar intake with k ($r=0.87$, $P < 0.0001$) and t_{50} ($r=-0.69$, $P=0.013$; Fig. 5B). Based on the significant and strong correlations, we developed a model predicting k from body mass and nectar intake as follows: $\log(k \text{ in } \text{h}^{-1}) = 0.9032 \times \log(\text{nectar intake in ml per day}) - 2.0307 \times \log(\text{body mass in g}) - 1.7617$ ($P < 0.001$, adjusted $R^2=0.89$).

DISCUSSION

After feeding, hummingbirds rapidly shift from using fat to fuel their metabolism to recently ingested sugar (Chen and Welch, 2014; Welch and Suarez, 2007) and synthesize fat for future needs (Eberts et al., 2019; Powers, 1991). Although hummingbirds have the capacity to rely on either glucose or fructose to completely fuel their metabolism, we demonstrated that when consumed together, hexoses can be directed to different metabolic fates. Additionally, we observed seasonal changes in fat turnover rates, with faster turnover rates in summer/breeding condition birds. We relate these to changes in fat stores and daily energy expenditure. To our knowledge, this is the first study to test for long-term metabolic partitioning of nutrients in birds, and it also illuminates how seasonal changes may influence the fat turnover rate.

Preferential use of glucose for *de novo* lipogenesis

Hummingbirds can rapidly deplete their fat stores and need to continuously rebuild them using foraged nectar. The higher $f_{\text{exo},\infty}$ parameter estimate for the ^{13}C enriched glucose trials indicates that a greater proportion of carbon from glucose was incorporated into the fat stores than carbon from fructose during labeled fructose trials. This suggests that glucose was preferentially used as a substrate for *de novo* lipogenesis and that there is metabolic partitioning of sucrose. Although hummingbirds have high sucrase activity and abundance of intestinal transporters for sugar uptake (Karasov et al., 1986), the proportion of paracellular to transporter mediated uptake increases with sugar concentration (McWhorter et al., 2006). As such, even if some dietary sugars are at least partially metabolized in the enterocytes via transport mediated uptake, it is likely that a majority of

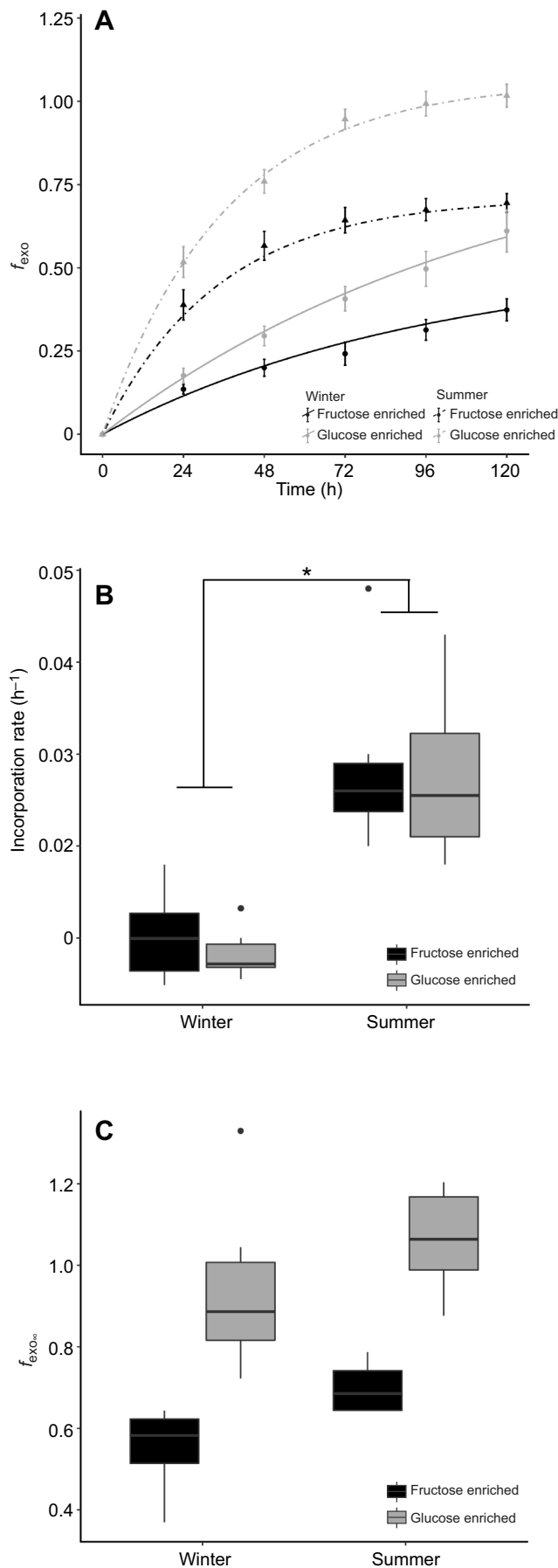


Fig. 3. Incorporation of dietary carbons into the fat stores of *A. colubris* hummingbirds over the course of 120 h estimated through fasting breath ^{13}C stable isotope signature. (A) Incorporation of ^{13}C enriched diets into fat stores modeled using a one-component exponential model. Fructose (black) and glucose (grey) are the proportion of expired CO_2 produced from the ^{13}C -enriched diets during the winter (triangles, solid lines) and summer (circles, dashed lines). Values are mean \pm s.e.m. Lines are the one-pooled models using the mean parameter estimates. (B,C) Parameter estimates from the one-pooled models for (B) rate of carbon incorporation, k , and (C) theoretical $f_{\text{exo,th}}$. The limits of the boxes represent the 25th and 75th percentiles, the bar the median, and the whiskers represent the maximum value, or 1.5 times the interquartile range. Circles represent outliers. Significant differences are denoted by * ($P < 0.05$).

dietary fructose and glucose is delivered intact to the splanchnic tissue. Potential drivers of hexose partitioning include an overall net preferential oxidation of fructose (Daly et al., 2000), which alters the amount and type of substrates available for fatty acid synthesis and the preference for glucose in the liver for fatty acid synthesis observed in rodent models (Carmona and Freedland, 1989). These two drivers are not mutually exclusive, and both could be occurring in hummingbirds. Additionally, there is building evidence to suggest that hummingbirds have the capacity for cellular uptake and oxidation of fructose in their flight muscles and heart observed through relatively high mRNA and protein of a fructose-specific transporter (GLUT5) in both tissues (Ali, 2018; Myrka and Welch, 2018; Welch et al., 2018). This adds additional potential routes for oxidation that are not available to mammals and could influence the rates at which fructose and glucose are oxidized to different fates. If extra-hepatic

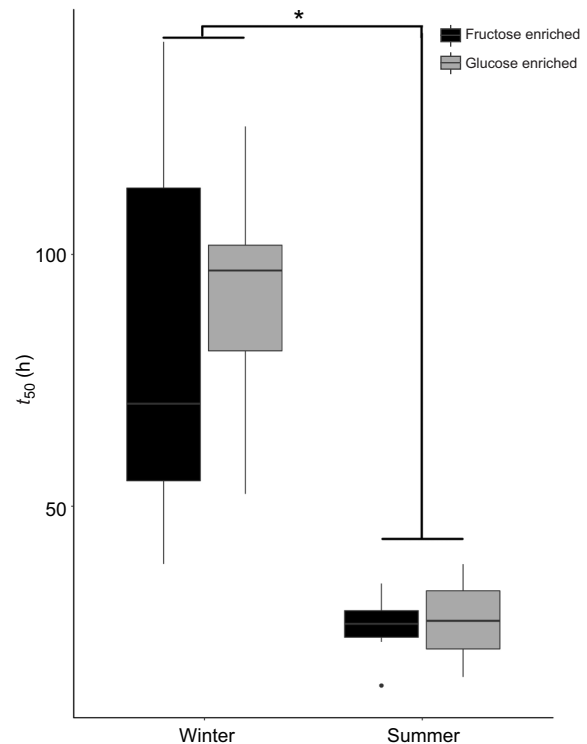


Fig. 4. Fat turnover rate (t_{50}) in ruby-throated hummingbirds (*A. colubris*) during the winter and summer. Hummingbirds were fed nectar diets with sucrose enriched with ^{13}C on the fructose (black) and glucose (grey) portions of the molecule. No significant effect of diet enrichment was found, and * indicates a significant seasonal effect ($P < 0.05$). The limits of the boxes represent the 25th and 75th percentiles, the bar the median, and the whiskers represent the maximum value, or 1.5 times the interquartile range. The circle represents an outlier. $n=6$ per season.

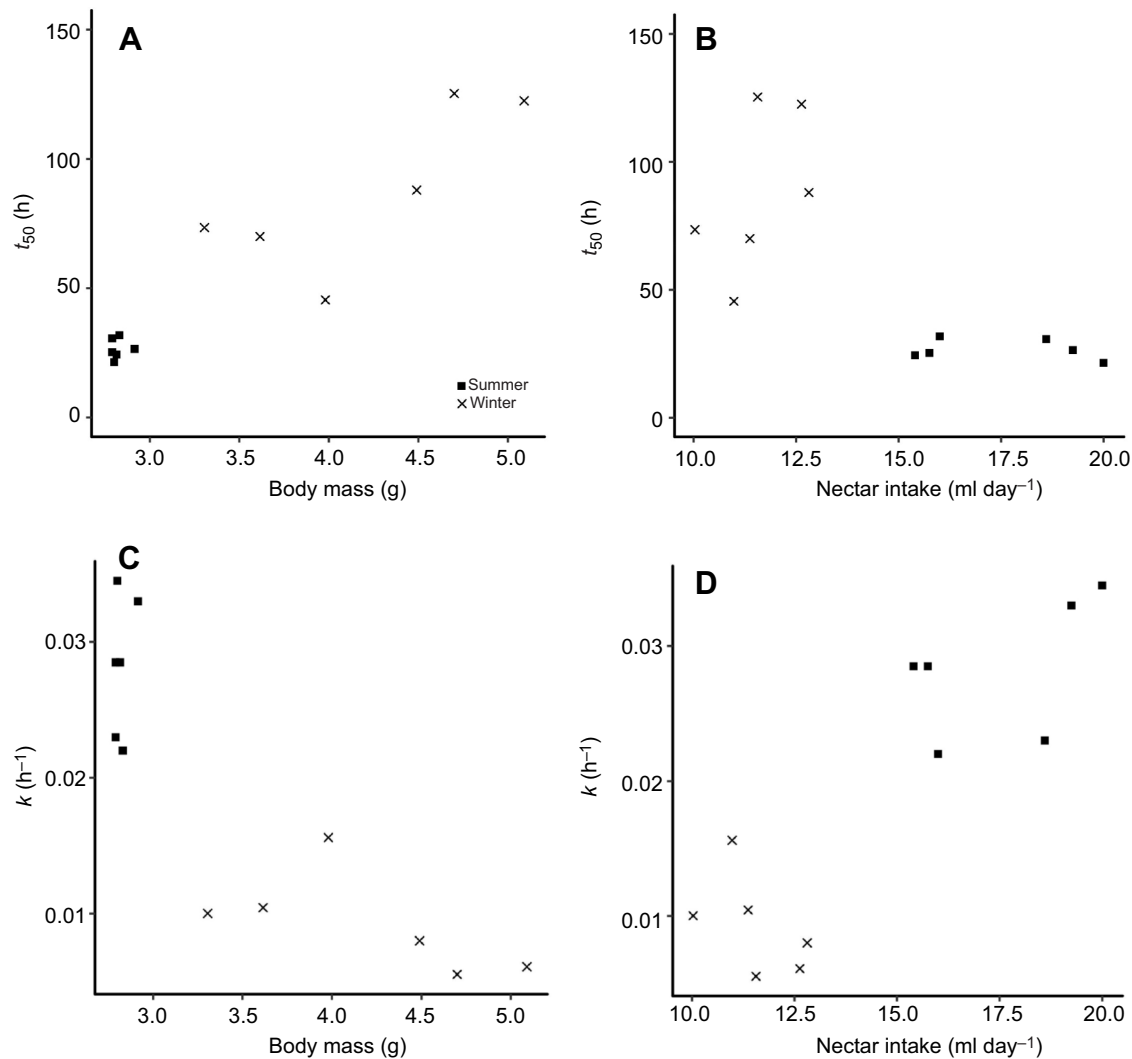


Fig. 5. Fat turnover rate is correlated with body mass and energy use in *A. colubris*. Relationship between fat turnover rate (t_{50}) and (A) body mass and (B) nectar intake, and relationship between k and (C) body mass and (D) nectar intake in ruby-throated hummingbirds (*A. colubris*). Values are averaged for the glucose and fructose trials for the winter (black crosses) and summer (black squares). $n=6$ per season.

tissues have limited or no ability to use fructose as a fuel, this may increase the proportion of fructose that is used for fatty acid synthesis in the liver and increase the proportion of glucose used for immediate oxidation.

The liver and other splanchnic tissues are considered to be the primary sites of fructose metabolism among well-studied vertebrates (Jang et al., 2018). The hummingbird liver has the capacity for both glucose and fructose uptake, and the abundance of ketohexokinase and aldose B suggests that fructose could be rapidly metabolized by the liver (Myrka and Welch, 2018). Ingested sugars are rapidly absorbed and taken up by the liver for fatty acid synthesis (Eberts et al., 2019), thus substrate preference in the liver could drive partitioning of dietary sugars. In rodent models, fatty acid synthesis rate may depend on dietary history and substrate (Carmona and Freedland, 1989). Elevated fructose intake increases the preference for glucose as a substrate for fatty acid synthesis compared with fructose (Carmona and Freedland, 1989; Chevalier et al., 1972; Romsos and Leveille, 1974).

The exact substrates used for fatty acid synthesis, in terms of direct hepatic uptake of glucose or fructose or uptake of metabolites derived from them (such as lactate), is unknown. We have

previously hypothesized that hummingbirds would preferentially use fructose for *de novo* lipogenesis and preserve glucose for direct oxidation in the flight muscles (Welch et al., 2018). The present study suggests that the opposite may be occurring. But partitioning of sucrose metabolism during flight has not yet been determined. Hummingbirds do spend a significant portion of time between foraging bouts perching while they digest their meal (Diamond et al., 1986). Because hummingbirds do not maintain substantial glycogen stores, the muscle's demand for glucose is likely dramatically reduced while perching. As such, hepatic preference for glucose as a substrate may drive how sugars are used. We are unable to determine what metabolic paths glucose and fructose enter upon absorption, and future studies incorporating ^{13}C metabolic flux analysis (Dai and Locasale, 2017) will provide key information to understand how glucose and fructose are used with the body to meet immediate energy needs and whether that is dependent on activity state.

Seasonal changes in fat turnover rate

We found seasonal differences in the t_{50} , with dramatically faster turnover rates in the summer. In summer, the t_{50} was just over a day

(~26 h) and similar to the time frames reported for broad-tailed hummingbirds (Carleton et al., 2006) and tent-making bats (O'Mara et al., 2017). Replacing half of their fat reserves in approximately 1 day suggests that these animals may be more at risk of starvation compared with larger birds or mammals (O'Mara et al., 2017). We predicted that we would observe seasonal differences in t_{50} . However, the fat turnover rates during the winter were longer than initially anticipated (t_{50} values exceeding 87 h or 3.6 days). Tissue turnover rates can be influenced by metabolic rate, energy expenditure, body mass and life stage (Brown, 2000; Carleton and Martinez del Rio, 2005; Keicher et al., 2017). Brown (2000) proposed that turnover rate is negatively related to body mass. Thus larger hummingbirds will have a longer turnover rate. However, energy demands such as thermoregulation and activity also influence turnover rate and could mask the relationship with body mass (Houlihan et al., 1995). However, this may be case and tissue dependent. Increasing metabolic rate via cold exposure did not alter the carbon turnover rate of red blood cells (Carleton and Martinez del Rio, 2005). Additionally, Bauchinger et al. (2010) found increased turnover rates in tissues with cold exposure but not with increased activity. They suggested that the increase in turnover rate during cold exposure was due to protein metabolism associated with tissue remodeling. Unlike these studies, our study focused on turnover rate of fat stores rather than structural or functional tissue turnover rates (red blood cells or flight muscles), which could influence the capacity for variability in turnover rate. Because adipose tissue more directly reflects turnover rate of fuel stores (energy expenditure and food consumption) compared with structural tissues, adipose tissue may have the highest degree in variability of turnover rate compared with other tissues.

The variation in t_{50} was strongly related to both body mass and daily nectar intake. Fat turnover rates were slower in heavier birds that had larger fat stores. Birds that consumed less nectar per day also had longer turnover rates. Using nectar intake as a proxy of daily energy expenditure (because mass change across time was minimal), animals that expended more energy had faster turnover rates. These seasonal differences in turnover rate are likely driven by the rate of input into the fat stores and the size of the fat stores, which consumption and daily energy expenditure would directly influence.

During the winter months, our hummingbirds consumed less nectar and were more sedentary than during the summer (M. F. Dick, personal observation). This could also contribute to the increase in body mass. In our captive colony, we observed seasonal trends in nectar intake. Nectar intake was higher during the summer months, followed by decreasing consumption in the winter. Consumption increased again during the spring molt and through to the end of summer (Fig. 6, data represent individuals from the present study and additional individuals). We do note that although body mass was constant in the summer, body mass decreased slightly during the winter trials (average 0.3 ± 0.03 g total over the 5 days, or 7% of the starting body mass). Carleton et al. (2006) noted that hummingbirds in a negative energy balance had longer fat turnover rates owing to lower rates of fat synthesis and deposition, and depletion of fat stores. This could have also contributed to the longer turnover rate during the winter in our birds.

Seasonal and environmental differences may also influence daily energy expenditure in wild hummingbirds. In broad-tailed hummingbirds, daily energy expenditure can range from 12.6 kJ during the dry season to 39.8 kJ in the wet season, despite body mass remaining constant (Shankar et al., 2019). In that study, variability in daily energy expenditure was largely attributed to changes in daily activity (time spent perching, flying and hovering). Animals were more active in the wet season when nectar availability was limited,

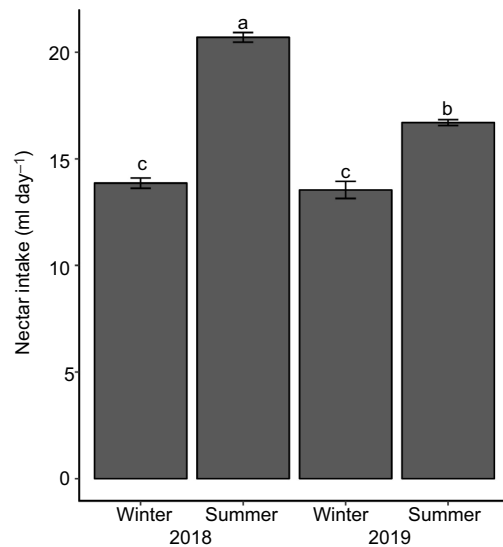


Fig. 6. Seasonal changes in nectar intake of captive ruby-throated hummingbirds (*A. colubris*) from 2018 to 2019. Hummingbirds include those in represented in the study and additional individuals from the colony. $n=9$ per season. Values represent seasonal means \pm s.e.m. Values that do not share a common letter differ.

which increased foraging time (Shankar et al., 2019). Our study confirms that seasonal changes in energy expenditure occurs in other hummingbird species and under controlled environmental conditions. Using nectar intake and composition, and assuming animals remain in energy balance, we predict a daily energy expenditure of approximately 22 kJ in the winter and 34 kJ in the summer for birds in our study. Using an allometric model from Nagy et al. (1999) to estimate hummingbird daily energy expenditure and the seasonal body masses of our birds, we would predict an energy intake of 31 kJ for winter and 16 kJ for summer. However, this model only accounts for body mass and not any innate variation in energy expenditure across the annual cycle. If increasing daily energy expenditure decreases t_{50} , animals may potentially be more vulnerable to starvation during these times. Our captive animals were in a stable abiotic environment. Wild animals may face considerably more daily variation in energy expenditure and intake, and thus fat turnover rate. For example, inclement weather may disrupt normal foraging behavior (Gass and Lertzman, 1980) or rapidly impose metabolic rate increases (e.g. increased thermoregulatory costs; Welch and Suarez, 2008). This may become especially important during migration, when fat stores fluctuate with migratory flight and refueling periods.

Conclusions

In summary, our study demonstrates metabolic partitioning of simple dietary sugars between immediate oxidation and *de novo* lipogenesis. Hummingbirds have the remarkable capacity to rely on either glucose or fructose to fuel their metabolism and have adaptations at multiple steps along the sugar oxidation cascade to enable high carbon flux rates. Although both glucose and fructose were used for lipogenesis, glucose was preferentially used for fatty acid synthesis over fructose. Although hummingbirds have the capacity to rely on either hexose, metabolic preference does exist and is an important feature in understanding the high sugar flux needed to fuel flight and lipogenesis. Furthermore, fat turnover may vary seasonally with changes in body mass and expenditure. This adds to a growing body of work informing us on factors that influence how animals manage their energy sources.

Acknowledgements

We thank the UTSC vivarium staff for caring for the hummingbirds, and the Welch Lab volunteers for their assistance in hummingbird capture.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.F.D., K.C.W.; Methodology: M.F.D., K.C.W.; Validation: M.F.D.; Formal analysis: M.F.D.; Investigation: M.F.D., A.A.-T., Y.S.O.; Resources: K.C.W.; Data curation: M.F.D.; Writing - original draft: M.F.D.; Writing - review & editing: M.F.D., A.A.-T., Y.S.O., K.C.W.; Visualization: M.F.D.; Supervision: K.C.W.; Funding acquisition: K.C.W.

Funding

This research was supported by a Natural Sciences and Engineering Research Council of Canada (NSERC) Discovery Grant (number 386466) and the Human Frontier Science Program (number RGP0062/2016). Key instrumentation was acquired through an NSERC Research Tools and Infrastructure grant (RTI-2016-00509).

References

- Ali, R. S. (2018). Plasma membrane recruitment patterns of glucose transporters (GLUT) 1, 2, 3, and 5 in response to feeding in the ruby-throated hummingbird, *Archilochus colubris*. MSc thesis, University of Toronto, Toronto, Canada.
- Bakdash, J. Z. and Marusich, L. R. (2017). Repeated measures correlation. *Front. Psychol.* **8**, 456. doi:10.3389/fpsyg.2017.00456
- Baker, H. G. and Baker, I. (1990). The predictive value of nectar chemistry to the recognition of pollinator types. *Isr. J. Plant Sci.* **39**, 157-166.
- Bates, D., Mächler, M., Bolker, B. and Walker, S. (2015). Fitting linear mixed-effects models using lme4. *J. Stat. Softw.* **67**, 1-48. doi:10.18637/jss.v067.i01
- Baty, F., Ritz, C., Charles, S., Brutsche, M., Flandrois, J.-P. and Delignette-Muller, M.-L. (2015). A toolbox for nonlinear regression in R: the package nlstools. *J. Stat. Softw.* **66**, 1-21. doi:10.18637/jss.v066.i05
- Blem, C. R. (1976). Patterns of lipid storage and utilization in birds. *Am. Zool.* **16**, 671-684. doi:10.1093/icb/16.4.671
- Brown, J.H., West, G.B. and Enquist, B.J. (2000). Scaling in biology: patterns, processes, causes, and consequences. In: *Scaling in Biology* (ed. J. H. Brown and G. B. West). Oxford University Press, New York.
- Carleton, S. A. and Martinez del Rio, C. (2005). The effect of cold-induced increased metabolic rate on the rate of 13 C and 15 N incorporation in house sparrows (*Passer domesticus*). *Oecologia* **144**, 226-232. doi:10.1007/s00442-005-0066-8
- Carleton, S. A., Bakken, B. H. and Martinez del Rio, C. (2006). Metabolic substrate use and the turnover of endogenous energy reserves in broad-tailed hummingbirds (*Selasphorus platycercus*). *J. Exp. Biol.* **209**, 2622-2627. doi:10.1242/jeb.02293
- Carmona, A. and Freedland, R. A. (1989). Comparison among the lipogenic potential of various substrates in rat hepatocytes: the differential effects of fructose-containing diets on hepatic lipogenesis. *J. Nutr.* **119**, 1304-1310. doi:10.1093/jn/119.9.1304
- Chen, C. C. W. and Welch, K. C. (2014). Hummingbirds can fuel expensive hovering flight completely with either exogenous glucose or fructose. *Funct. Ecol.* **28**, 589-600. doi:10.1111/1365-2435.12202
- Chevalier, M. M., Wiley, J. H. and Leveille, G. A. (1972). Effect of dietary fructose on fatty acid synthesis in adipose tissue and liver of the rat. *J. Nutr.* **102**, 337-342. doi:10.1093/jn/102.3.337
- Dai, Z. and Locasale, J. W. (2017). Understanding metabolism with flux analysis: From theory to application. *Metab. Eng.* **43**, 94-102. doi:10.1016/j.ymben.2016.09.005
- Daly, M. E., Vale, C., Walker, M., Littlefield, A., George, K., Alberti, M. M. and Mathers, J. (2000). Acute fuel selection in response to high-sucrose and high-starch meals in healthy men. *Am. J. Clin. Nutr.* **71**, 1516-1524. doi:10.1093/ajcn/71.6.1516
- Diamond, J. M., Karasov, W. H., Phan, D. and Carpenter, F. L. (1986). Digestive physiology is a determinant of foraging bout frequency in hummingbirds. *Nature* **320**, 62. doi:10.1038/320062a0
- Eberts, E. R., Dick, M. F. and Welch, K. C. (2019). Metabolic fates of evening crop-stored sugar in ruby-throated hummingbirds (*Archilochus colubris*). *Diversity* **11**, 9. doi:10.3390/d11010009
- Fisher, H. I. and Bartlett, L. M. (1957). Diurnal cycles in liver weights in birds. *Condor* **59**, 364-372. doi:10.2307/1365247
- Gass, C. L. and Lertzman, K. P. (1980). Capricious mountain weather: a driving variable in hummingbird territorial dynamics. *Can. J. Zool.* **58**, 1964-1968. doi:10.1139/z80-270
- Groom, D. J. E., Bayram, N., Shehata, M., Herrera, M. L. G. and Welch, K. C. (2019). Low ambient temperature reduces the time for fuel switching in the ruby-throated hummingbird (*Archilochus colubris*). *Comp. Biochem. Physiol. A Mol. Integr. Physiol.* **237**, 110559. doi:10.1016/j.cbpa.2019.110559
- Hiebert, S. M. (1991). Seasonal differences in the response of rufous hummingbirds to food restriction: body mass and the use of torpor. *Condor* **93**, 526-537. doi:10.2307/1368184
- Hou, L. and Welch, K. C. (2016). Premigratory ruby-throated hummingbirds, *Archilochus colubris*, exhibit multiple strategies for fuelling migration. *Anim. Behav.* **121**, 87-99. doi:10.1016/j.anbehav.2016.08.019
- Houlihan, D. F., Carter, C. G. and McCarthy, I. D. (1995). Protein turnover in animals. In *Nitrogen Metabolism and Excretion* (ed. P. Walsh and P. Wright), pp. 1-32. Boca Raton, FL: CRC Press.
- Jang, C., Hui, S., Lu, W., Cowan, A. J., Morscher, R. J., Lee, G., Liu, W., Tesz, G. J., Birnbaum, M. J. and Rabinowitz, J. D. (2018). The small intestine converts dietary fructose into glucose and organic acids. *Cell Metab.* **27**, 351-361. doi:10.1016/j.cmet.2017.12.016
- Karasov, W. H., Phan, D., Diamond, J. M. and Carpenter, F. L. (1986). Food passage and intestinal nutrient absorption in hummingbirds. *Auk* **103**, 453-464. doi:10.1093/auk/103.3.453
- Keicher, L., O'Mara, M. T., Voigt, C. C. and Dechmann, D. K. N. (2017). Stable carbon isotopes in breath reveal fast metabolic incorporation rates and seasonally variable but rapid fat turnover in the common shrew (*Sorex araneus*). *J. Exp. Biol.* **220**, 2834-2841. doi:10.1242/jeb.159947
- Kleiber, M. (1975). Metabolic turnover rate: a physiological meaning of the metabolic rate per unit body weight. *J. Theor. Biol.* **53**, 199-204. doi:10.1016/0022-5193(75)90110-1
- Lighton, J. (2008). *Measuring Metabolic Rates: A Manual for Scientists: A Manual for Scientists*. Oxford University Press.
- McCue, M. D. and Welch, K. C. (2016). ¹³C-Breath testing in animals: theory, applications, and future directions. *J. Comp. Physiol. B* **186**, 265-285. doi:10.1007/s00360-015-0950-4
- McWhorter, T. J., Bakken, B. H., Karasov, W. H. and Martinez del Rio, C. (2006). Hummingbirds rely on both paracellular and carrier-mediated intestinal glucose absorption to fuel high metabolism. *Biol. Lett.* **2**, 131-134. doi:10.1098/rsbl.2005.0388
- Myrka, A. M. and Welch, K. C., Jr (2018). Evidence of high transport and phosphorylation capacity for both glucose and fructose in the ruby-throated hummingbird (*Archilochus colubris*). *Comp. Biochem. Physiol. B Biochem. Mol. Biol.* **224**, 253-261. doi:10.1016/j.cbpb.2017.10.003
- Nagy, K. A., Girard, I. A. and Brown, T. K. (1999). Energetics of free-ranging mammals, reptiles, and birds. *Annu. Rev. Nutr.* **19**, 247-277. doi:10.1146/annurev.nutr.19.1.247
- O'Mara, M. T., Wikelski, M., Voigt, C. C., Ter Maat, A., Pollock, H. S., Burness, G., Desantis, L. M. and Dechmann, D. K. (2017). Cyclic bouts of extreme bradycardia counteract the high metabolism of frugivorous bats. *elife* **6**, e26686. doi:10.7554/eLife.26686
- Powers, D. R. (1991). Diurnal variation in mass, metabolic rate, and respiratory quotient in Anna's and Costa's hummingbirds. *Physiol. Zool.* **64**, 850-870. doi:10.1086/physzool.64.3.30158211
- Romsos, D. R. and Leveille, G. A. (1974). Effect of dietary fructose on *in vitro* and *in vivo* fatty acid synthesis in the rat. *Biochim. Biophys. Acta Lipids Lipid Metab.* **360**, 1-11. doi:10.1016/0005-2760(74)90175-1
- Shankar, A., Graham, C. H., Canepa, J. R., Wethington, S. M. and Powers, D. R. (2019). Hummingbirds budget energy flexibly in response to changing resources. *Funct. Ecol.* **33**, 1904-1916. doi:10.1111/1365-2435.13404
- Suarez, R. K. (1992). Hummingbird flight: sustaining the highest mass-specific metabolic rates among vertebrates. *Experientia* **48**, 565-570. doi:10.1007/BF01920240
- Suarez, R. K., Brownsey, R. W., Vogl, W., Brown, G. S. and Hochachka, P. W. (1988). Biosynthetic capacity of hummingbird liver. *Am. J. Physiol. Reg I* **255**, R699-R702. doi:10.1152/ajpregu.1988.255.5.R699
- Tieszen, L. L., Boutton, T. W., Tesdahl, K. G. and Slade, N. A. (1983). Fractionation and turnover of stable carbon isotopes in animal tissues: implications for $\delta^{13}\text{C}$ analysis of diet. *Oecologia* **57**, 32-37. doi:10.1007/BF00379558
- Voigt, C. C., Matt, F., Michener, R., and Kunz, T.H. (2003). Low turnover rates of carbon isotopes in tissues of two nectar-feeding bat species. *J. Exp. Biol.* **206**, 1419-1427. doi:10.1242/jeb.00274
- Welch, K. C. and Suarez, R. K. (2007). Oxidation rate and turnover of ingested sugar in hovering Anna's (*Calypte anna*) and rufous (*Selasphorus rufus*) hummingbirds. *J. Exp. Biol.* **210**, 2154-2162. doi:10.1242/jeb.005363
- Welch, K. C. and Suarez, R. K. (2008). Altitude and temperature effects on the energetic cost of hover-feeding in migratory rufous hummingbirds, *Selasphorus rufus*. *Can. J. Zool.* **86**, 161-169. doi:10.1139/Z07-127
- Welch, K. C., Myrka, A. M., Ali, R. S. and Dick, M. F. (2018). The metabolic flexibility of hovering vertebrate nectarivores. *Physiology* **33**, 127-137. doi:10.1152/physiol.00001.2018