



Low ambient temperature reduces the time for fuel switching in the ruby-throated hummingbird (*Archilochus colubris*)

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ABSTRACT

Physiological adaptations that enhance flux through the sugar oxidation cascade permit hummingbirds to rapidly switch between burning lipids when fasted to burning ingested sugars when fed. Hummingbirds may be able to exert control over the timing and extent of use of ingested sugars by varying digestive rates when under pressure to accumulate energy stores or acquire energy in response to heightened energy demands. We hypothesized that hummingbirds would modulate the timing of a switch to reliance on ingested sugars differently when facing distinct energetic demands (cool versus warm ambient temperatures). The timing of the oxidation of a single nectar meal to fuel metabolism was assessed by open-flow respirometry, while the time to first excretion following the meal was used as a proxy for digestive throughput time. As predicted, birds showed a more rapid switch in respiratory exchange ratio (RER = rate of O₂ consumption/CO₂ production) and excreted earlier when held at cool temperatures compared to warm. In both cases, RER peaked barely above 1.0 indicating ingested sugar fueled ≈ 100% of resting metabolism. Our findings suggest that energetic demands modulate the rate of fuel switching through shifts of the sugar oxidation cascade. The speed of this shift may involve decreases in gut passage times which have previously been thought to be inflexible, or may be caused by changes in circulation as a result of low ambient temperature.

1. Introduction

Hummingbirds have some of the highest mass-specific metabolic rates among vertebrates, in part due to their small size and their reliance upon energetically expensive hovering flight while foraging (Suarez, 1992). Unsurprisingly, hummingbirds possess a range of physiological, morphological, and behavioral adaptations that allow them to supply substrates, particularly sugar, to their working muscles in order to support this high rate of energy expenditure. These adaptations for high rates of sugar flux, from ingestion to oxidation, allow for the rapid shift from use of endogenously stored fat to newly ingested carbohydrates within minutes following the intake of a nectar meal (Chen and Welch, 2014; Suarez et al., 1990; Welch Jr. et al., 2007; Welch et al., 2006). The immediate and direct oxidation of ingested sugars to fuel metabolism recovers roughly 15% more energy compared to first converting these sugars to endogenous lipids, then followed by oxidation (Suarez et al., 2011, 1990). Immediate oxidation of ingested

nectar, then, unlocks the maximum potential net energy from this fuel and can more rapidly be utilized as an oxidative substrate than if it were first used for lipogenesis. This also allows for the preservation of stored fat in preparation for migration or during environmentally challenging conditions. The ability to continuously supply sugars from ingested nectar to sites of catabolic activity, at rates fast enough to fully sustain foraging behaviour, relies on a suite of behavioral and physiological specializations comprising the “sugar oxidation cascade” (Suarez et al., 2011). As a multistep process, flux of carbon from nectar to tissue mitochondria is enabled by sufficient flux through each and every step of the cascade. Conversely, variation in, and constraints on, overall flux through the sugar oxidation cascade may be attributable to any number of steps, though some steps likely exert more control than others.

One way to gain insight into the dynamics of fuel selection is to examine fuel switching under different energetic conditions, as higher energetic demands may lead to more rapid fuel switching. If there is excess capacity across the entire sugar oxidation cascade, or

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hummingbirds are able to modulate various steps of the cascade flexibly, then hummingbirds may demonstrate more rapid fuel switching when energetic demands are high. In this study we examine the role of energetic demands upon the rate of fuel switching, as elicited by cold exposure, to see if the timing of fuel switching can decrease under heightened metabolic rates.

Being among the first steps of the sugar oxidation cascade, digestive throughput may, in part, set the rate of fuel switching. Investigations into hummingbird digestive transit time suggests that foraging frequency closely matches crop emptying rates (Diamond et al., 1986; Karasov et al., 1986), indicating that digestive throughput may be a major limitation to the upregulation of flux through the sugar oxidation cascade and thus the timing of the switch from lipid to sugar oxidation. However, there is evidence that hummingbirds are able to modulate their foraging frequency and nectar intake, suggesting that digestive throughput is plastic and may depend on a variety of both intrinsic and extrinsic factors. For example, hummingbirds possess the ability to modulate foraging frequency and meal size in order to modify net energy accumulation to match variable ongoing energy demands (Gass et al., 1999), or to build energy stores more or less rapidly to meet changing energy needs associated with fasting (Hiebert, 1991; Tooze and Gass, 1985), breeding/care of young (Calder et al., 1990), or migratory flight (Carpenter et al., 1983, 1993; Hiebert, 1993; Hou et al., 2015; Hou and Welch Jr., 2016). Overall, this suggests that hummingbirds have excess digestive capacity and may be able to modulate the flux through the sugar oxidation cascade with changes in digestive throughput, depending on metabolic and environmental contexts. However, any kind of measurement on how digestive performance relates to the maximum flux through the sugar oxidation cascade has not been performed in hummingbirds.

Here, we examine how metabolic demand shapes the sugar oxidation cascade and the timing for the shift from oxidation of endogenously stored fat to newly ingested sugars. As they are among the smallest vertebrate endotherms, thermoregulation represents a significant challenge for hummingbirds. Low ambient temperatures are associated with dramatic increases in basal (Lasiewski, 1963), perching (Chai et al., 1998; López-Calleja and Bozinovic, 1995), and even hovering metabolic rate (Chai et al., 1998; Schuchmann, 1979; Welch Jr. and Suarez, 2008). Hummingbirds faced with the need to meet elevated metabolic demands at lower ambient temperatures have been shown to dramatically increase foraging effort in response (Fernández et al., 2002; Gass et al., 1999; Hou and Welch Jr., 2016). Thus, we predicted that this more rapid shift to oxidizing newly ingested sugar would be achieved, at least in part, by an overall increase in digestive throughput rate. We also predicted that hummingbirds would switch from oxidizing endogenous lipids to oxidizing sugar from a single newly ingested meal more rapidly when held at low ambient temperature (13 °C) compared to a higher ambient temperature (26 °C), due to the higher energy demands of thermoregulation.

2. Materials and methods

Animal husbandry and all experiment procedures conformed with the Canadian Council on Animal Care and were approved by the University of Toronto Animal Care Committee.

2.1. Animal capture and husbandry

Experiments were conducted on male ruby-throated hummingbirds (*Archilochus colubris*; $n = 8$; mass range: 2.45–3.77 g) captured on the campus of the University of Toronto Scarborough. Birds were housed individually in metal EuroCages (Corners Limited, Kalamazoo, Michigan, USA) measuring 91.5 cm W × 53.7 cm H × 50.8 cm D at the University of Toronto Scarborough vivarium at 20 °C. While housed, hummingbirds were offered a diet of 20% (w/v) Nektar Plus (Guenter Enderle, Tarpon Springs, FL, USA) solution. This diet was provided ad

libitum to birds in 10-ml syringes that were replaced daily. The hummingbirds' daylight schedule approximated the light-dark cycle they would naturally encounter in their environment, including photoperiod that approximated those they would naturally encounter as part of their annual migratory journey from Toronto to central Mexico and back.

2.2. Experimental design

To explore how both variation in ambient temperature and time of day affected the use of ingested fuel, we developed a two-by-two factorial experimental design. Hummingbirds are known to demonstrate diel patterns of foraging rates, with high rates in the morning and early evening compared to the mid-day (Calder et al., 1990). To account for any variation with time of day, all hummingbirds were tested in the morning (1–6 h after lights on) and in the afternoon (6–11 h after lights on). Ambient temperature was held at 13 °C (13.3 ± 0.1 °C; mean \pm s.e.m.) or at 26 °C (26.1 ± 0.0 °C) inside an environmental chamber in which metabolic chambers were housed. All hummingbirds were tested at each of the four temperature and time of day combinations, with at least 2 weeks between any two experimental runs with a given individual. All birds were held in their home cages at 20 °C at all times, except when undergoing an experimental trial (~2 h per trial). Data were collected between October 2015 and March 2016.

Hummingbirds underwent a training period approximately one month before data collection began to acclimatize them to metabolic chambers and hand-feeding of experimental meals. Body mass of hummingbirds was measured before and after placement in metabolic chambers. Individuals were held inside a metabolic chamber for a total of approximately 2 h. A small wooden dowel near the bottom of the chamber served as a perch for the birds. At the beginning of a training or experiment period, an individual bird was removed from housing and placed inside a metabolic chamber, without food. Birds were fasted inside the metabolic chamber for approximately 1 h to ensure that they had cleared food from their crop and that they were primarily or exclusively oxidizing fat. Following the 60-minute fasting period within the chamber, the lid to the chamber was removed and the bird was fed 50 μ l of a 20% sucrose solution. All birds ingested the entire 50 μ l meal immediately when it was offered. The exact timing of food administration was noted within the respirometry data recording, and were visible on video records (see below). Birds remained in the chamber for approximately 65 min following ingestion of the meal.

2.3. Respirometry and video recording

Rates of oxygen consumption and carbon dioxide production were obtained via open flow chamber respirometry. The flow of ambient air through both the metabolic chamber containing the hummingbird and a reference, empty chamber was controlled via a Flowbar-8 mass flow controller (Sable Systems International, Las Vegas, NV, USA). Air flow through the metabolic chamber was maintained at 400–500 ml/min at all times. Excurrent air from the chambers was subsampled at a steady flow rate of 200–250 ml/min. Sequential subsampling of either the focal metabolic chamber or the reference empty chamber was controlled by a RM-8 flow multiplexer (Sable Systems International). Subsampled air was passed through a water vapour pressure meter, carbon dioxide meter, a drying column (Indicating Drierite, W.A. Hammond Drierite, Xenia, Ohio, USA), and finally an oxygen analyzer (Turbofox-5, Sable Systems International). The oxygen and carbon dioxide analyzers were regularly calibrated according to manufacturer instructions using well-mixed ambient air for the oxygen analyzer, and zero and 0.25% CO₂ reference gases for the CO₂ analyzer. Video of the bird inside the metabolic chamber was recorded simultaneously with respirometry data to confirm that birds remained calm within the chamber and to capture any excretion events (see below).

Respirometry data were recorded at a frequency of 1 Hz using Expedata software (v. 1.84, Sable Systems) starting approximately

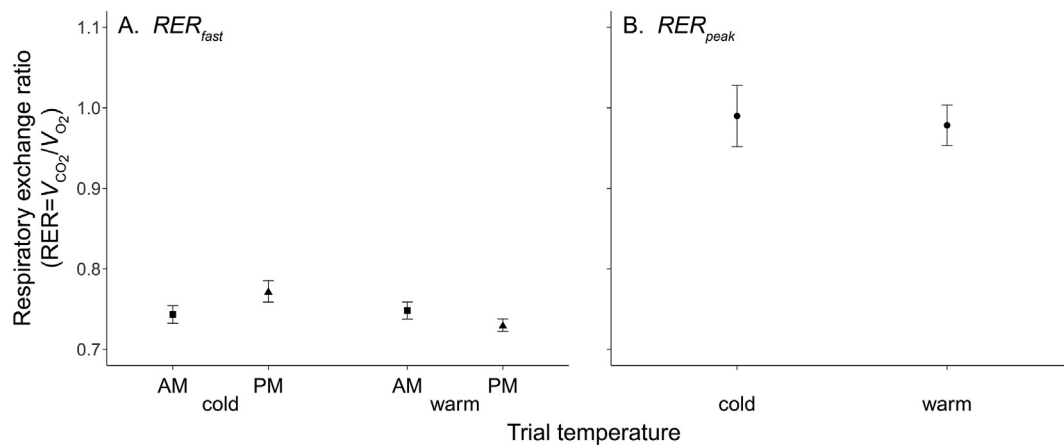


Fig. 1. Mean respiratory exchange ratios ($RER = \dot{V}_{CO_2} / \dot{V}_{O_2}$) displayed by ruby-throated hummingbirds (*A. colubris*) during A) the final ~20 min of fasting prior to being fed a single 50 μ l sugar water meal (RER_{fast}) and B) the peak values reached after feeding on 50 μ l of sugar water (RER_{peak}). Birds were tested at two ambient temperatures: cold, 13 °C, and warm, 26 °C. Trial temperature had no significant effect on RER values. The interaction of trial temperature and time of day was significant for RER_{fast} only ($P = .038$).

20 min prior to feeding the bird. Data were recorded for 3 min while sampling from the empty reference chamber, followed by two 7.5-minute recording periods from the chamber holding a bird. Each measurement of the bird chamber was separated by a 2-minute recording period from the reference chamber. After this 20-minute period, subsampling was resumed from the reference chamber for 3 min, during which the chamber containing the bird was opened and the bird was fed. After feeding, subsampling was continued from the chamber containing the bird, continuing for three 19-minute periods, each separated by 2-minute reference chamber recordings. A final 3-minute sampling of the reference chamber was started and the bird was removed from its chamber, approximately 65 min after it had been fed, the video recording was stopped, and the bird was weighed and returned to the vivarium.

2.4. Data analysis

The exact timing of feeding of hummingbirds varied slightly (≤ 3 min difference among recordings) depending on operator handling. As the timing of feeding was noted in recordings, all analyses were henceforth referenced to the precise feeding time of a given experimental run. Raw gas measurements were drift and lag-corrected and rates of oxygen consumption (\dot{V}_{O_2} , in ml/min) and carbon dioxide production (\dot{V}_{CO_2} , in ml/min) were calculated from these corrected raw data by application of standard equations (Lighton, 2008; Welch Jr., 2011). We then calculated respiratory exchange ratios ($RER = \dot{V}_{CO_2} / \dot{V}_{O_2}$). Data were binned into 2-minute intervals with mean values calculated for all variables within each bin.

To quantify how metabolic rate and RER varied over time and among trials we first calculated the average RER values observed during the ~20 min of fasting prior to the feeding event (RER_{fast}). We also calculated the peak RER value observed after feeding for each trial (RER_{peak}) as the largest RER binned value found within the first 30 min following feeding. The time to peak RER ($time_{RER_{peak}}$, in min) was taken as the time at which peak RER occurred relative to feeding time. We also calculated the average metabolic rate over the approximately 20-minute period prior to feeding ($\dot{V}_{O_2_{fast}}$, in ml O_2 /h) as well as the metabolic rate observed at the time point when the maximum RER value was observed ($\dot{V}_{O_2_{peak}}$, in ml O_2 /h). We converted instantaneous readings of metabolic rate to their oxyjoule equivalent (MR_W , in W) using the following equation (modified from Lighton, 2008) with the " \dot{V}_{O_2} " term converted to units of ml O_2 /s,

$$MR_W = \dot{V}_{O_2} \times [16 + 5.164(RER)] \quad (1)$$

And integrated these values over the period of time from feeding to peak RER to determine the total amount of energy expended during each trial over this period ($E_{fast-peak}$, in kJ).

In order to gain insight into gut passage times, we examined video recordings of birds and noted how soon each individual first excreted after feeding ($time_{excrete}$, in minutes). While this approach does not allow us to quantify the passage of the entirety of the previously ingested meal nor assess mean meal retention time (sensu Karasov et al., 1986), it was not possible for us to video-record each individual indefinitely following feeding. Nonetheless, we assume that $time_{excrete}$ correlates well with overall gut passage time of an ingested meal and the timing of availability of ingested sugars to tissues as an oxidative fuel following absorption across the intestinal brush border.

To control for the effect of time of day, we modelled time of day and ambient temperature, as well as their potential interaction, on each of the parameters listed above using a mixed effect modeling approach with individual ID included as a random effect. However, time of day consistently failed to show a significant effect on any parameter. Further, the interaction of time of day and temperature was significant for only one dependent variable: RER_{fast} . Model comparison using corrected AIC scores also showed strongest support for models with only temperature included as a fixed effect (data not shown). Thus, except for RER_{fast} , only temperature and individual (as a random factor) were included in models fit to all other data (meaning two experimental runs were included at each temperature for each individual). An $\alpha = 0.05$ was used throughout. Data are presented as means \pm s. e. m.

3. Results

Fasted, perching ruby-throated hummingbirds displayed respiratory exchange ratios (RER_{fast}) that were between 0.71 and 0.75 during the approximately 20-minute period prior to feeding (Fig. 1A). When time of day was included as a factor, the mixed effect model indicated the time of day \times temperature interaction was significant ($P = .038$; Fig. 1A) while RER_{fast} values did not significantly differ as a function of either factor alone ($P \geq .069$). RER_{fast} values were slightly lower early in the day (1–6 h after lights on: 0.74 ± 0.01) compared to later in the day (6–11 h after lights on: 0.77 ± 0.01), when the bird was tested at 13 °C, while the pattern was reversed when birds were tested at 26 °C (1–6 h after lights on: 0.75 ± 0.01 ; 6–11 h after lights on: 0.73 ± 0.01). When time of day was excluded as a factor, RER_{fast} values did not differ significantly as a function of ambient temperature ($P = .1140$) and averaged 0.76 ± 0.01 and 0.74 ± 0.01 for 13 °C trials and 26 °C trials, respectively.

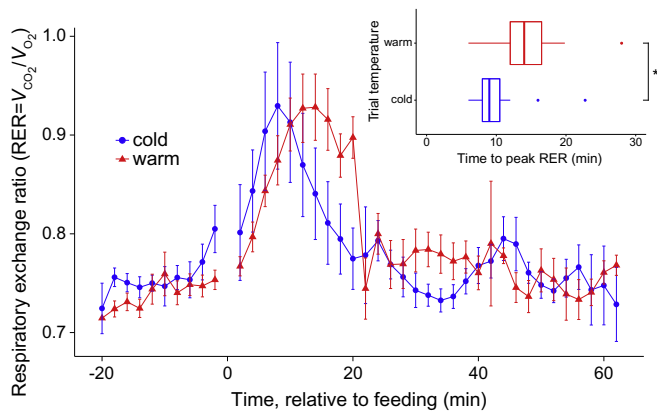


Fig. 2. Variation in respiratory exchange ratios ($RER = \dot{V}_{CO_2}/\dot{V}_{O_2}$) exhibited by ruby-throated hummingbirds relative to the timing of ingestion (time = 0) of a single 50 μ l sugar water meal. Data are binned every 2 min and averaged across individuals within a temperature trial (cold, 13 °C, and warm, 26 °C). The inset shows the time to peak RER values ($time_{RERpeak}$ in min, relative to feeding). On average, $time_{RERpeak}$ was longer during warm trials compared to cold trials ($P = .007$).

The time of day during which birds were tested (AM versus PM) had no effect on any of the other response variables examined. As time of day was not a significant predictor for any variables assessed, and because, aside from RER_{fast} , the interaction of time of day and temperature were not significant predictors of any other variables, we re-ran all models after dropping “time of day” as a fixed effect. Results from the simpler models (including temperature as a fixed effect and bird ID as a random effect) are presented hereafter.

Respiratory exchange ratios began rising shortly after hummingbirds were fed (Fig. 2). In both cold and warm temperature trials, RER values (RER_{peak}) peaked at approximately 1 (Fig. 1B; 13 °C trials: 0.99 ± 0.04 ; 26 °C trials: 0.98 ± 0.03 ; $P = .771$). However, the timing of the peak, relative to feeding ($time_{RERpeak}$), varied significantly with trial temperature ($P = .007$). $time_{RERpeak}$ averaged 10.3 ± 1.0 and 14.8 ± 1.5 min, during 13 °C and 26 °C trials, respectively (Fig. 2, inset).

Hummingbird oxygen consumption rates were significantly higher during 13 °C compared to 26 °C trials (Fig. 3a; $P = .0118$) and tended to be higher during the 20 min prior to feeding (\dot{V}_{O_2fast}) compared to values obtained at the time at which RER values peaked (\dot{V}_{O_2peak}), though this difference was not significant ($P = .0510$). There was no significant interaction of trial temperature and dietary state (fasted versus peak RER values; $P = .581$). \dot{V}_{O_2fast} values averaged 57.9 ± 2.6 and 44.0 ± 3.3 ml O_2/h for 13 °C and 26 °C trials, respectively, while \dot{V}_{O_2peak} values averaged 50.4 ± 2.5 and 40.5 ± 2.3 ml O_2/h for 13 °C and 26 °C trials, respectively. RER and \dot{V}_{O_2} values were used to calculate metabolic rate (in W) using eq. 1 above. Similar to oxygen consumption rate, hummingbird metabolic rate (MR_W) was, on average, significantly higher during trials at 13 °C compared to those at 26 °C trials (Fig. 3b; $P = .0061$). Like oxygen consumption rates, metabolic rates also tended to be higher during the 20 min prior to feeding (MR_{Wfast}) compared to values obtained at the time at which RER values peaked (MR_{Wpeak}), though this effect was even weaker, and not significant (Fig. 3b; $P = .2000$). MR_{Wfast} averaged 0.32 ± 0.01 and 0.24 ± 0.01 W at 13 °C and 26 °C, respectively, while MR_{Wpeak} averaged 0.30 ± 0.01 and 0.24 ± 0.01 W at 13 °C and 26 °C, respectively.

As birds expended energy at a more rapid rate during cold trials, but the time between feeding and the shift to complete reliance on oxidation of the newly ingested sugar meal to support metabolism (i.e. $time_{RERpeak}$ values) was shorter, we quantified the total amount of energy each bird expended between the time they were fed and when RER_{peak} values were reached ($E_{fast-peak}$). On average, birds expended similar amounts of energy (i.e. exhibited similar $E_{fast-peak}$ values) from

feeding to the time of peak RER values during both 13 °C and 26 °C trials (Fig. 4; 13 °C trials: 1.29 ± 0.15 kJ; 26 °C trials: 1.63 ± 0.20 kJ; $P = .122$).

In most cases, we definitively identified the first excretion event captured on video following feeding and determined the elapsed time since the bird was fed ($time_{excrete}$, in minutes). However, no urination event was observed during the video recording period in three trials at 26 °C, involving three birds (#74, 76, and 77). These instances were treated as missing data and models were run as described above. Average $time_{excrete}$ was significantly longer during 26 °C trials, compared to 13 °C (Fig. 5; 13 °C trials: 18.2 ± 1.9 min; 26 °C trials: 32.9 ± 6.1 min; $P = .001$).

4. Discussion

Ruby-throated hummingbirds are among the smallest vertebrate endotherms and the temperature range examined in this study was below the thermoneutral zone of similarly sized hummingbirds (López-Calleja and Bozinovic, 1995). As expected, there was an increase in metabolic rate associated with greater thermogenic demand at 13 °C compared to 26 °C. We also observed that oxygen consumption rates of perching ruby-throated hummingbird averaged over the final ~20 min of fasting (\dot{V}_{O_2fast}) tended to be higher than oxygen consumption rates measured when birds exhibited maximum RER values following feeding, (\dot{V}_{O_2peak}), though this difference was not statistically significant ($P = .0510$). Some of this difference is due to variation in the amount of oxygen consumed per mole of ATP produced depending on which class (carbohydrate versus lipid) of metabolic substrate is oxidized (reviewed in Brand, 2003, 2005). Indeed, hovering hummingbirds consume roughly 15–18% more oxygen to generate a given amount of ATP from the complete oxidation of pure lipids, compared to sole utilization of carbohydrates glucose or glycogen (Welch Jr. et al., 2007). In fact, the difference in \dot{V}_{O_2fast} and \dot{V}_{O_2peak} averaged 11% and 14% for trials at 13 °C and 26 °C, respectively. The differences in \dot{V}_{O_2fast} and \dot{V}_{O_2peak} values observed in this study provide further support for the contention that differences in biochemical stoichiometry are apparent in whole animal oxygen consumption measurements. However, when metabolic rate is expressed as watts, we do not see any significant difference in metabolic rate between \dot{V}_{O_2fast} and \dot{V}_{O_2peak} (Fig. 3b). This suggests that the energetic costs did not differ before and after a meal, indicating that the cost of processing a single nectar meal is small.

Similar to previous studies (Chen and Welch, 2014; Welch Jr. et al., 2007; Welch et al., 2006), we found that fasted hummingbirds display RER values approaching 0.7, indicating primary reliance on endogenous lipids as an oxidative fuel. As observed previously (Chen and Welch, 2014; Welch Jr. et al., 2007; Welch et al., 2006), RER rapidly rose following feeding and peaked at values very near, or equal to 1.0, indicating birds had shifted to oxidizing newly ingested sugars primarily or exclusively to fuel their perching metabolism. While previous studies noted the rapidity of this shift towards reliance on newly ingested sugar as an oxidative fuel, this is the first study to correlate variation in the timing of this shift to differences in ambient temperature. The quicker onset of RER_{peak} and excretion timing, and the higher metabolic rates related to lower temperatures suggest that metabolic demand increases the rate of flux through the sugar oxidation cascade, potentially through decreases in digestive transit times.

Hummingbirds are noted for their ability to dynamically regulate food intake, such as when acquiring energy for fasting (Hiebert, 1991; Tooze and Gass, 1985), preparing for migration (Carpenter et al., 1993; Carpenter et al., 1983; Hiebert, 1993; Hou et al., 2015; Hou and Welch Jr., 2016), and during the breeding season (Calder et al., 1990; Hou et al., 2015). Such variation suggests the possibility of dynamic regulation of crop emptying or digestive throughput as well. Many species of birds show variation in crop emptying rates, in large part due to the energy or nutrient density of the meal, dietary make-up, and the volume

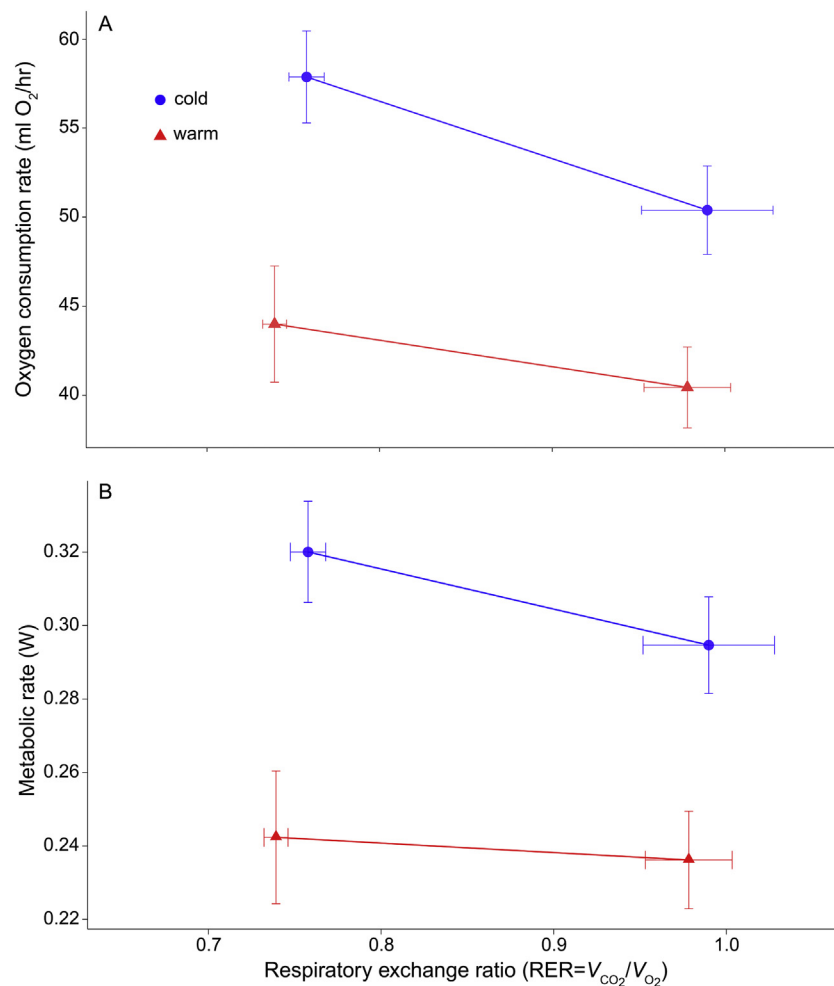


Fig. 3. Average \dot{V}_{O_2} consumption rate (\dot{V}_{O_2} in ml O_2 /h; panel A) and metabolic rate (MR_W in W; panel B) in perching ruby-throated hummingbirds during the final 20 min of fasting prior to being fed a single 50 μ l sugar water meal ($\dot{V}_{O_2,fast}$; $MR_{W,fast}$) and at the time when respiratory exchange ratio (RER) values peaked following feeding ($\dot{V}_{O_2,peak}$; $MR_{W,peak}$). Birds were tested at two ambient temperatures: cold, 13 °C, and warm, 26 °C. \dot{V}_{O_2} and MR_W values were significantly higher in birds under cold, compared to warm, conditions ($P = .0118$ and $P = .0061$, respectively). $\dot{V}_{O_2,fast}$ values tended to be higher than $\dot{V}_{O_2,peak}$ values, though this difference was not statistically significant ($P = .0510$). While the same directional trend was true for MR_W , the effect was weaker ($P = .2000$).

of food consumed (Brown and Downs, 2003; López-Calleja et al., 1997; Witmer, 1998). For example, some passerines decrease transit times with decreasing sugar concentrations (Downs, 1997; Witmer, 1998), while others modify transit time depending on the type of food ingested (insect versus nectar versus fruit) (Brown and Downs, 2003). More complex or difficult to digest foods may require slower gut transit durations to ensure energy extraction. Thus, variation in transit time, and presumably crop emptying rates, may be an adaptive response when dietary intake changes (Price et al., 2015). As a result, gut function has been implicated in limiting energy intake, and putting animals into negative energy balance, when facing constitutively high rates of energetic expenditure (McWhorter and Martínez del Río, 2000). Here, we show that hummingbirds excrete earlier in the cold, suggesting a link between rates of energetic expenditure and digestive motility.

$Time_{excrete}$ is not a traditional measure of digestive function from ingestion to elimination from the colon, as most of the dietary water in hummingbirds is voided through the kidneys (Martínez del Río et al., 2001; McWhorter and del Río, 1999). But, it does provide some indication of how rapidly the nectar meal was assimilated and absorbed across the brush-border of the intestine. Earlier excretion may be a result of more rapid digestive transit times, as faster throughput of ingested nectar will likely induce an earlier onset of osmotic disturbance of the nectar meal, requiring an earlier $time_{excrete}$. It is unknown if

increased metabolic rates influence kidney function and renal water loss, as the increase in cardiac output that accompanies elevated resting metabolic rates may also explain the quicker onset of excretion. However, hummingbirds are capable of reducing glomerular filtration rates when undergoing a fast and stop excreting to prevent dehydration (Bakken et al., 2004). Thus, any change in $time_{excrete}$ may not be related to changes in metabolism per se, but rather to the time it takes for the kidneys to respond to changes in osmotic balance associated with the single meal. Furthermore, research comparing fasted Swainson's thrush (*Catharus ustulatus*) at rest to those flown in a wind tunnel has found that GFR does not change between these conditions (Gerson and Guglielmo, 2013), suggesting that metabolic rate does not influence GFR. Overall, this suggests that kidney function and excretion may not be directly related to metabolic rate. To our knowledge, kidney function under different temperature regimes has not been measured in hummingbirds, and it is unknown how environmental conditions influence kidney function. This is an avenue for future research.

Increased cardiac output in hummingbirds at colder temperatures may also enhance carbohydrate absorption in the gut. Sugar absorption across the intestinal brush border membrane is both an active and passive process, with glucose and fructose absorbed by cellular-mediated and paracellular pathways in hummingbirds and many other volant organisms (Caviedes-Vidal et al., 2007; McWhorter et al., 2006; Price et al., 2015). As a result, the difference in concentration of glucose

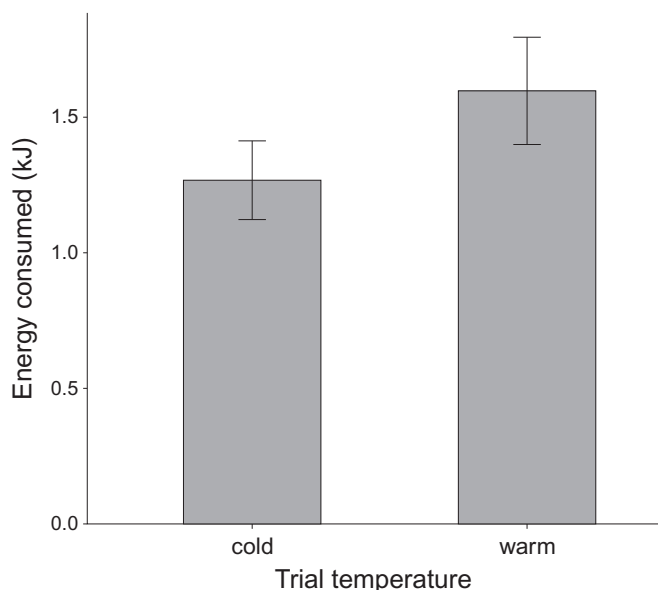


Fig. 4. Energy consumed (in kJ) by perching ruby-throated hummingbirds from the time of ingesting a single 50 μ l sugar water meal to the point at which each individual's respiratory exchange ratio (RER) values peaked following feeding. Similar amounts of energy were consumed by birds tested at two ambient temperatures: cold, 13 $^{\circ}$ C, and warm, 26 $^{\circ}$ C ($P = .122$).

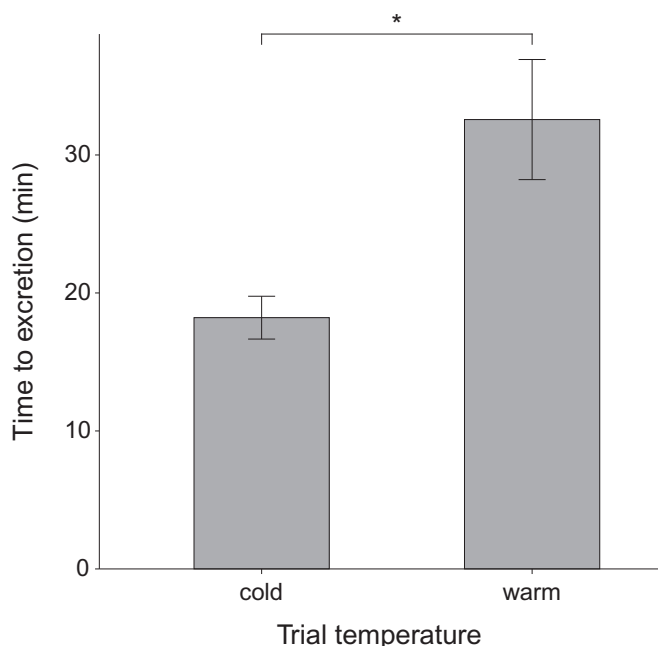


Fig. 5. Average time to first excretion event (in min) in perching ruby-throated hummingbirds fed a single 50 μ l sugar water meal tested at two temperatures, 13 $^{\circ}$ C and 26 $^{\circ}$ C. Time to excretion was significantly longer for birds held in warm, compared to cold, conditions ($P = .001$).

and fructose between the intestinal lumen and the extracellular space lining the basal membrane of the brush border plays a role in determining absorption rate. Higher cardiac output, coupled with a high capacity for glucose and fructose uptake by tissues (Chen and Welch, 2014; Welch et al., 2006), may facilitate the rapid sugar uptake across the intestine via passive transport by maintaining low glucose concentrations at the intestinal vasculature.

Another possible explanation for the shift in fuels may be related to the availability of circulating fatty acids. Poultry species demonstrate

increases in circulating insulin when transitioning from the fasted to fed state (Anthony et al., 1990). While birds are largely insulin-insensitive with respect to blood glucose regulation (Braun and Sweazea, 2008), their adipose tissue may respond to increased levels of insulin with a reduction in lipolysis and fatty acid mobilization (Duncan et al., 2007). This reduction in lipolysis due to feeding results in a large decline in circulating non-esterified fatty acids (Anthony et al., 1990). Due to the higher metabolic rate of the cold treated birds compared to the warm, cold birds may consume the remaining fatty acids in circulation faster, and subsequently transition to glucose once the circulating fatty acids are depleted. However, because no measures of circulating fatty acid levels are available, we avoid further speculation on this possibility.

Overall, the rate of fuel switching in hummingbirds is influenced by metabolic rate, but there are multiple mechanisms that may explain the more rapid fuel switching when facing cold stress in hummingbirds, all of which may contribute to the speed of fuel switching. A reductionist approach into the different steps of the sugar oxidation cascade may provide insight into which aspects are limiting.

We are unsure what the importance is of the significant interaction between temperature and time of day upon RER_{fast} . All birds were fasted for 1 h and should have reached fasting blood sugar levels, and have largely switched to fat as their primary fuel, as indicated by an RER ranging from 0.73–0.77. Given that the effect size of time of day and temperature was low, we suggest that the effect of time of day upon fasted RER may be spurious, and does not reflect a true physiological phenomena.

Mass-specific \dot{V}_{O_2} values were calculated to facilitate comparison with published data presented in this format. Mass-specific \dot{V}_{O_2peak} values measured in 26 $^{\circ}$ C trials averaged 13.4 ± 1.1 ml O_2 $g^{-1} h^{-1}$ in our birds, a value similar to that reported by Chai et al. (1998) for ruby-throated hummingbirds perch-feeding at 25 $^{\circ}$ C. However, mass-specific \dot{V}_{O_2peak} values measured at 13 $^{\circ}$ C in our birds (17.2 ± 0.8 ml O_2 $g^{-1} h^{-1}$) are lower than those predicted at 13 $^{\circ}$ C (~ 26 ml O_2 $g^{-1} h^{-1}$) by the regression equation of perch-feeding metabolic rate versus temperature reported by Chai and Dudley (1998). However, unlike our \dot{V}_{O_2peak} data, which are from individuals fed a single sugar water meal, the \dot{V}_{O_2} values reported by Chai and Dudley (1998) were obtained from perch-feeding birds that were allowed to feed at will throughout the experiment. The act of feeding likely incurs a small metabolic cost above resting rates. More importantly, particularly at 13 $^{\circ}$ C where the discrepancy between our data and that predicted by Chai and Dudley is greater, the difference likely has to do with the increased thermogenic demands associated with warming the cool nectar upon ingestion (i.e. during each perch-feeding respirometric measurement; Lotz et al., 2003). Birds in our study only had to warm a single, small sugar water meal. All birds tested by Chai et al. (1998) were feeding at the moment oxygen consumption measures were taken, and paid the cost to warm nectar each time they fed (and provided data). Clearly, the calculated thermal conductance (slope of the regression line) reported by Chai and Dudley is greater than that we would calculate based on data presented here, and is likely due to the greater conductance associated with ingestion of cooler nectar (Lotz et al., 2003). However, given that our data were collected at only two temperatures, we are hesitant to place too much weight on calculations of thermal conductance.

5. Conclusion

Hummingbirds have high rates of energy turnover, due to their small size and flight style (Suarez, 1992), so efficient energy acquisition and the maintenance of energy balance for their daily needs is paramount. When facing high energetic demands, rapid fuel switching from fats to carbohydrates is favored to preserve stored fat, because the use of newly ingested carbohydrates can generate up to 15% more energy rather than converting it to fat before catabolism (Suarez et al., 1990). Here, we found that hummingbirds make this switch faster when under

energetically demanding conditions, facilitated by a more rapid upregulation of flux through the sugar oxidation cascade. This upregulation may be related to digestive throughput, but increased transportation capacity associated with high heart rate or reductions in available circulating fatty acids may also contribute to the faster shifting in fuels.

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Declaration of Competing Interest

The authors have no conflict of interests to declare.

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