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# Evidence of high transport and phosphorylation capacity for both glucose and fructose in the ruby-throated hummingbird (*Archilochus colubris*)

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## ABSTRACT

Hummingbirds are able to fuel hovering flight entirely with recently ingested glucose or fructose. Among vertebrates, several steps of sugar flux from circulation to skeletal muscle are potentially rate-limiting, including transport into muscle and subsequent phosphorylation. While capacities for glucose flux are substantial, capacities for fructose flux are comparatively low. The mechanisms underlying apparent high rates of glucose and fructose oxidation in hummingbird flight muscle remain unclear. We examined relative expression of facilitative sugar transporters (GLUTs) and enzymes of fructolysis in ruby-throated hummingbird (*Archilochus colubris*) tissues involved in energy homeostasis and flight, *via* qPCR and measured hexokinase activity in pectoralis *in vitro*. We hypothesized that expression of these genes was upregulated in hummingbird flight muscle compared to other vertebrates. We found that hummingbird pectoralis had high relative transcript abundance of GLUT1 and GLUT5 compared to expression profiles of other vertebrates. In particular, GLUT5 expression in pectoralis was similar to that of intestine. We demonstrated minimal relative densities of fructolytic enzymes in pectoralis, suggesting that the ketohexokinase pathway does not rapidly metabolize fructose in these muscles. Instead, we found that the capacity for phosphorylation of either glucose or fructose by hexokinase is very high in pectoralis *in vitro*. The contributions of individual hexokinase isoforms remain to be determined. Our results further characterize the strategies by which hummingbirds, and perhaps other nectarivores, accomplish rapid sugar flux. High transport and sugar phosphorylation capacities appear to exist in flight muscle, though the enzymatic pathways that catalyze the phosphorylation of sugar in muscle remain uncertain.

## 1. Introduction

Maintenance of energy homeostasis is a challenge faced by all life. The metabolic rates of small nectarivores, including bats and hummingbirds, are among the highest observed in vertebrates (Suarez, 1992). As a consequence, these animals face especially great challenges to energy homeostasis. In order to fuel their hovering activity on a nectar diet, hummingbirds have evolved the ability to power their foraging activity entirely using recently ingested sugar (Chen and Welch, 2014). This ability is made possible by a suite of adaptations enhancing flux of sugars from the intestine through circulation to actively oxidizing tissues. For example, birds generally exhibit much higher blood glucose levels than comparably sized mammals (Braun and Sweazea, 2008), and hummingbirds exhibit the highest blood glucose concentrations observed among birds (Beuchat and Chong, 1998), a feature that helps ensure high glucose availability to active tissues.

Hummingbirds and nectar bats have the ability to fuel hovering flight with recently ingested sugar, and some individual hummingbirds can fuel up to 100% of their aerobic activity this way (Chen and Welch, 2014; Welch et al., 2006, 2008; Welch and Suarez, 2007). The muscles of most well-characterized vertebrates, such as humans (Sun and Empie, 2012) and mice (Patel et al., 2015), do a poor job of taking up and phosphorylating fructose, with most fructose being delivered to the liver for conversion into other metabolites for subsequent circulation. This process delays the availability of fructose-derived calories to working muscle (Parks et al., 2008; Sun and Empie, 2012). Little is known about the abilities of avian muscles to metabolize fructose. When rates of sugar flux from ingestion to exhalation of labelled carbon were examined, these nectarivores did not appear to discriminate between glucose, fructose, or sucrose (Chen and Welch, 2014; Welch et al., 2006, 2008; Welch and Suarez, 2007). When hummingbirds were given any one of these sugars, there was no significant difference in the time taken for ingested carbon to appear in exhaled breath, or in the

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time for that trace to disappear from the breath when the birds were switched to an unlabeled sugar solution (Chen and Welch, 2014). This mirrors the hummingbird diet, as the monosaccharide content of the nectar favoured by these animals is roughly 50% fructose and 50% glucose (Baker et al., 1998). Given their fructose-rich diet, it is possible that, like for glucose, hummingbirds experience high blood fructose spikes after feeding. However, blood fructose concentrations have not yet been examined in these animals.

Following absorption of sugar from the gut, which involves high rates of both cell-mediated and paracellular transport across the brush border (Karasov, 2017) and absorption into circulation, three steps are believed to be potentially limiting to sugar metabolism in skeletal muscle. The first step is the delivery of sugar from the bloodstream to the target extracellular tissues at a sufficient rate (Step 1). This is enhanced in hummingbirds by high rates of sugar flux to the muscle extracellular environment, which is facilitated by high cardiac output (Bishop, 1997), extensive vascularity in working muscle (Mathieu-Costello et al., 1992), and high capacities for paracellular diffusion from the lumen of capillaries to the extracellular space (Vock et al., 1996).

Transmembrane movement of sugars does not occur passively through the lipid bilayer (Uldry and Thorens, 2004), necessitating facilitated transport of glucose and fructose. Transport capacity, defined by transporter abundance and function, thus comprises the second potentially rate-limiting step (Step 2). Facilitated sugar transport is accomplished by multiple isoforms of glucose transporters (GLUTs), a subset of which are shown in Table 1, along with human expression patterns. Qualitative GLUT isoform distribution in chickens is similar to that of mammals for GLUT1, GLUT2, GLUT3, and GLUT5 (Byers et al., 2017). In mammals, multiple GLUT isoforms are responsible for uptake of glucose into peripheral tissue, including skeletal muscle. GLUT1 is a ubiquitous, dedicated glucose transporter (Uldry and Thorens, 2004). GLUT4 is translocated to the sarcolemma from intracellular vesicles in response to insulin and other factors such as increased exercise (OsorIo-Fuentealba et al., 2013; Shepherd and Kahn, 1999). Lastly, relatively low densities of GLUT3, another widely expressed glucose transporter (Uldry and Thorens, 2004) are present in mammalian muscle as well. Both GLUT1 and GLUT3 transcripts have been found in all ruby-throated hummingbird tissues yet examined (Welch et al., 2013), but the relative abundance of the transcripts and proteins, and the contribution of individual GLUT isoforms to rapid sugar transport in hummingbird tissue, remain largely unknown. GLUT4 is absent from the genomes of all bird species that have yet been examined, and blood glucose levels in birds are unresponsive to physiologically relevant concentrations of insulin (Braun and Sweazea, 2008; Carver et al., 2001; Seki et al., 2003; Sweazea and Braun, 2006). How birds regulate blood sugar and how the different GLUT isoforms may be involved in regulation remain poorly understood (Braun and Sweazea, 2008).

Most characterized fructose transport is accomplished by GLUT2, which transports both glucose and fructose, and GLUT5, a dedicated fructose transporter (Uldry and Thorens, 2004). While it is the norm for glucose to be rapidly transported into working muscle by multiple GLUT isoforms (Uldry and Thorens, 2004), capacities for fructose uptake in mammalian muscle are comparatively low, and handled only by one GLUT isoform, GLUT5 (Gaster et al., 2000). GLUT5 is present at lower levels than GLUT1 and GLUT3 in mammalian muscle, and maintains a low transport capacity for fructose (Gaster et al., 2000). As with mammalian muscle, GLUT2 transcript was not found in ruby-throated hummingbird pectoralis (Welch et al., 2013). Given stable isotope tracking results that are difficult to explain if the pectoralis does not rapidly uptake fructose (Chen and Welch, 2014), we predicted that a fructose transporter must be present in pectoralis in high abundance and with rapid transport capacity. Previous work has not tested for the presence of GLUT5 in hummingbirds, but as GLUT2 transcript was absent from muscle (Welch et al., 2013), GLUT5 was a likely candidate for rapid fructose uptake in ruby-throated hummingbird muscle.

Following uptake of glucose or fructose into the cells of the target tissue, phosphorylation traps the sugar within the cell, directing it to a catalytic fate (Step 3; Wilson, 2003). In examined mammal species, hexokinases (HKs) (Enzyme 1; Fig. 1) phosphorylate glucose, but do not phosphorylate fructose at substantial rates because of comparatively low affinity for fructose as a substrate and low fructose availability stemming from comparatively low levels of circulating fructose and low transport capacity (Cárdenas et al., 1998). Measured maximal activity of hummingbird hexokinases with glucose *in vitro* is sufficient to match estimated rates of glucose flux through glycolysis (assuming glucose, and not glycogen, is the sole substrate) during hovering in primary flight muscle *in vivo* (Suarez et al., 2009).

The bulk of phosphorylation of ingested fructose has historically been thought to occur in the liver in characterized vertebrates (Cirillo et al., 2009), with a lesser contribution from the kidneys (Björkman and Felig, 1982), although recent evidence has demonstrated a role for the brain in fructose metabolism as well (Oppelt et al., 2017; Park et al., 2017). Hepatic fructose phosphorylation occurs through the fructolytic enzymes of the ketohexokinase pathway, which directs end products into steps five and six of glycolysis (Fig. 1; Sun and Empie, 2012). Low amounts of these fructolytic enzymes are found in mammalian muscle, but do not make a large overall contribution to phosphorylation of ingested fructose (Cirillo et al., 2009). The first two steps of fructolysis, catalyzed by ketohexokinase (KHK) and Aldolase B (AldoB) respectively (Enzymes 7 and 8; Fig. 1), are rate-limiting to hepatic fructose metabolism in characterized vertebrates (Bode et al., 1980). HK activity has not been previously assayed in hummingbirds using fructose as a substrate. If hummingbirds have evolved the capacity for relativity rapid fructolysis in flight muscle then we expect to find enhanced activity of these crucial, rate-limiting fructolytic enyzmes. Two isoforms of KHK are known in mammals; however, only one isoform has been found in birds, including Anna's hummingbirds (*Calypte anna*), for which the genome has been sequenced (NCBI GenBank), and a ruby-throated hummingbird (*Archilochus colubris*) liver transcriptome (Workman et al., 2017). The expression of KHK has not been previously examined in hummingbird tissue.

The behaviour of hummingbirds challenges the standard vertebrate model of fructose metabolism. As described above, hummingbirds can fuel their hovering behaviour just as rapidly with fructose as they can with glucose (Chen and Welch, 2014). This is not what one would expect if fructose metabolism to facilitate muscle contraction requires more than twice as many enzymatic steps as needed for dietary glucose due to uptake of fructose, and subsequent release of other metabolites, by the liver (Sun and Empie, 2012). We hypothesized that hummingbird flight muscles express higher levels of GLUT1 and GLUT5 than other vertebrates examined to facilitate rapid uptake of glucose and fructose, respectively. Suarez et al. (2009) have demonstrated that phosphorylation of glucose by HKs has the capacity to occur rapidly in rufous hummingbirds (*Selasphorus rufus*), but little is known about how hummingbirds might metabolize fructose to fuel exercise (Welch and Chen, 2014). Whether the fructolysis pathway is enhanced in hummingbird flight muscle has not been previously determined. We

**Table 1**
Glucose transporters — distribution and substrates in characterized mammals (*sensu* Zhao and Keating, 2007). Main tissues of expression are restricted to those investigated in this study. Approximate $K_m$ values for human proteins were obtained from Uldry and Thorens (2004). The $K_m$ presented for GLUT3 was obtained using 2-deoxyglucose. N/A indicates $K_m$ values too high to serve a major function at physiological concentrations of sugar.

| Isoform | $K_m$ (mM) for glucose | $K_m$ (mM) for fructose | Main tissues | Preferred substrate |
|---|---|---|---|---|
| GLUT1 | 3 | N/A | Everywhere | Glucose |
| GLUT2 | 17 | 76 | Liver, intestine, kidney | Glucose and fructose |
| GLUT3 | 1.4 | N/A | Brain | Glucose |
| GLUT4 | 5 | N/A | Muscle, heart | Glucose |
| GLUT5 | N/A | 6 | Intestine, kidney | Fructose |

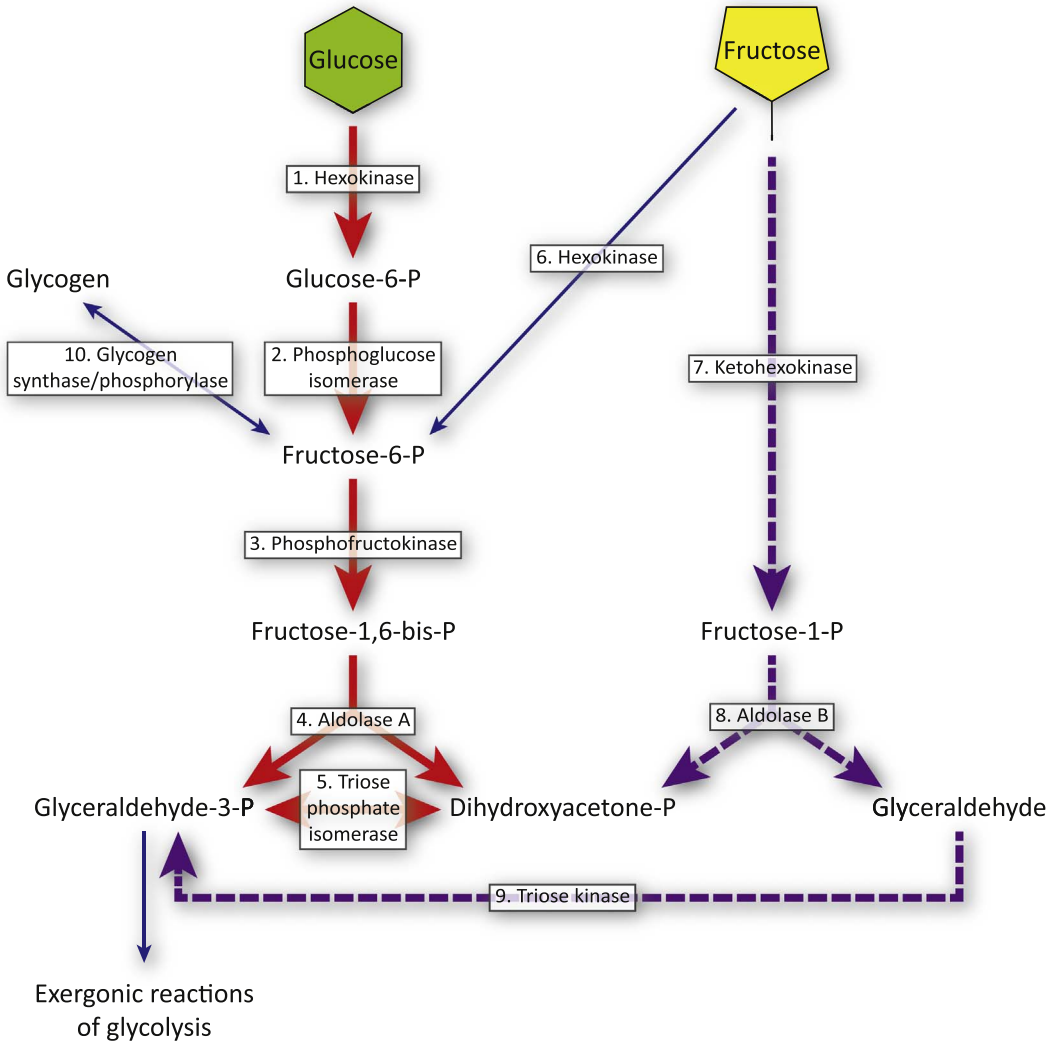


**Fig. 1.** Metabolic pathways of glucose and fructose prior to the exergonic reactions of glycolysis. The endergonic reactions of glycolysis are indicated by thick, solid arrows and the reactions of fructolysis are indicated by thick, dashed arrows. Adapted from Sun and Empie (2012) under the terms of the Creative Commons Attribution License.

hypothesized that hummingbird flight muscles express relatively higher levels of the enzymes KHK and AldoB than the muscles of other vertebrates that have been examined. We performed quantitative real-time PCR analysis (qPCR) on GLUT1, GLUT5, KHK, and AldoB using a variety of tissues from ruby-throated hummingbirds (*Archilochus colubris*). We predicted that relative transcript abundance of these genes would be uniquely high in hummingbird pectoralis when compared to other characterized vertebrate muscles. After locating a commercially available AldoB antibody that cross-reacted with ruby-throated hummingbird protein, we employed western blots and densitometry to compare AldoB protein expression across tissues. We predicted that protein abundance would be similarly high to transcript abundance in pectoralis. Finally, following analysis of KHK and AldoB, we assayed the enzyme activity of hummingbird endogenous HKs using either glucose or fructose substrate, to examine whether muscle HKs have the capacity to play a dominant role in fructose phosphorylation in hummingbirds. We hypothesized that if fructolysis could not account for apparent rapid fructose oxidation, then a hummingbird HK would, by necessity, display unusually high catalytic activity using fructose as a substrate.

## 2. Methods

### 2.1. Ethics statement and animal handling

The protocols for this study were approved by and performed in accordance with the requirements of the University of Toronto Animal Care Committee and the Canadian Wildlife Service Permit (CA 0258).

Wild adult male ruby-throated hummingbirds (*Archilochus colubris*) were captured at the University of Toronto Scarborough using modified box traps. Birds were housed in the University of Toronto Scarborough vivarium and fed NEKTON-Nectar-Plus (Nekton, Tarpon Springs, FL, USA) *ad libitum*. Birds were sacrificed after *ad libitum* feeding by an overdose of isoflurane followed by asphyxiation with helium between March 2014 and June 2015. Samples of pectoralis, liver, heart, brain, whole intestine, kidneys, and ankle flexor and extensor muscles were collected immediately after sacrifice. No attempt was made to separate supracoracoideus from pectoralis due to technical difficulty and uniform fiber type in these muscles (Welch and Altshuler, 2009). Mouse liver tissue (*Mus musculus*, strain C57BL/6J) was flash frozen and stored at −80 °C immediately following euthanasia for another research project taking place at the University of Toronto Scarborough.

### 2.2. DNA and RNA quality control

Purity and integrity of nucleic acids was assessed after each step of isolation or synthesis. Purity was evaluated by 260 nm/280 nm and 260 nm/230 nm UV absorbance using a NanoDrop 1000 (Thermo Scientific, Waltham, Massachusetts). Integrity was assessed using 1% agarose gel electrophoresis.

### 2.3. RNA isolation and cDNA synthesis

Immediately following tissue collection, RNA was extracted using Tri Reagent protocol (Sigma Aldrich Corporation, St. Louis, Missouri) with the following modifications: up to 100 mg of tissue was used per 1 ml of Tri Reagent, depending on the mass of available tissue. Tissues were homogenized using an RNase free glass tissue homogenizer and RNase free syringes of increasing needle gauge. Homogenized samples were stored at − 80 °C for up to four weeks and thawed on ice for 90 min prior to further processing. Phase extraction and pellet washing with ethanol were performed three times per sample. DNase I digestion and first strand cDNA synthesis were performed immediately afterwards.

RNA samples were digested with Deoxyribonuclease I, Amplification Grade (Life Technologies), as per the manufacturer's directions. First strand cDNA synthesis from whole RNA was performed using SuperScript III First-Strand Synthesis Supermix for qRT-PCR (Life Technologies) as per the manufacturer's directions.

### 2.4. Primer design

MEGA 5.2.2 with ClustalW alignment was used to construct a multiple sequence alignment for Elf1α1 in mRNA from avian sequences available in NCBI GenBank (National Center for Biotechnology Information, Bethesda, Maryland). An area of high homology across birds, located in the interior of the mRNA sequences, was chosen for primer design using Geneious 7.0.6 (geneious.com). NCBI Primer-BLAST (www.ncbi.nlm.nih.gov/) was used to check for non-specific amplicons within the “Aves” taxid (8782).

Primers were designed for GLUT1, GLUT5, KHK, and AldoB, using Anna's Hummingbird (*Calypte anna*) sequences, which became available during the course of this study (GenBank accession no. **XM_008503695.1**, **XM_008503671.1**, **XM_008492930.1**, and **XM_008496441** respectively).

Primer specificity and optimal primer annealing temperature were determined by temperature-gradient PCR. A genomic DNA template control was used to assess specificity for cDNA.

Efficiency of specific primer sets was assessed by amplification of a dilution series of hummingbird liver cDNA. Liver cDNA was used for primer testing because all genes of interest were expected to be stably expressed in this tissue. qPCR of this dilution series was performed as described below. The dilution series started at cDNA derived from approximately 0.05 ng/μl of whole RNA and included five more ten-fold dilutions. Dilution factor was plotted against $C_t$ value and analyzed by linear regression in Microsoft Excel (Microsoft, Albuquerque, New Mexico, USA). An efficiency of 90–105% was considered satisfactory. Primers used in this study are presented in Table 2.

### 2.5. qPCR assays

#### 2.5.1. Reaction conditions

Real time PCR was performed in the Center for the Neurobiology of Stress at the University of Toronto Scarborough, in triplicate, using SYBR green chemistry. A Bio-Rad PTC-200 Peltier Thermal Cycler and a Chromo 4 Continuous Fluorescence Detector were used in conjunction with Bio-Rad Opticon Monitor 3 software (Bio-Rad Laboratories, Hercules, California). 20 μl reactions were performed with IQ SYBR Green Supermix (Bio-Rad). Primers were included at a final concentration of 0.5 μM each and template was added to a final concentration of 0.05 ng/μl. The program began with one cycle of 94 °C for 4 min, followed by 40 cycles of 94 °C for 30 s, 60 °C for 1 min and 72 °C for 1 min 30 s. A melting curve was immediately performed from 45 to 95 °C. $C_t$ values observed in no reverse transcriptase controls were two to three orders of magnitude greater than those seen in corresponding cDNA samples, suggesting that genomic contamination amounted to < 1% of available template in cDNA samples, which was considered negligible.

#### 2.5.2. Data analysis

$C_t$ values of triplicates were averaged for each gene in each tissue of each bird. $C_t$ values were then compared using the delta-delta $C_t$ method. Elf1α1 was used to normalize the data. Following log transformation of the data, GLUT1, GLUT5, KHK, and AldoB were examined independently and the tissue with the lowest expression of each was chosen as the calibrator for that gene (liver for GLUT1 and brain for GLUT5, KHK, and AldoB). Calibration was achieved by dividing all normalized tissue measurements by that of the averaged value of the calibrator tissue. A linear mixed effects model, with the calibrator tissue set as the intercept, and Tukey HSD Post-hoc tests were performed to assess differences in transcript abundance among tissues independently for each of the four genes of study. A P-value of $< 0.05$ was considered significant. Statistical analyses were performed in R ver. 3.1.2 (R Foundation for Statistical Computing, Vienna, Austria).

### 2.6. Western blotting

Samples of pectoralis, liver, heart, brain, ankle flexor and extensor muscles, whole intestine, and kidneys were collected. Due to small tissue mass, we did not attempt to remove intestinal contents. One sample of frozen mouse liver was obtained as described above and included as a positive control. Samples were homogenized in RIPA buffer (50 mM Tris HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.1% SDS, 1% Triton X-100, 1 mM DTT) with Sigma-Aldrich Protease Inhibitor Cocktail for use with mammalian cells and tissue extracts, in DMSO solution. Samples were then incubated at 4 °C with agitation for 2 h and the homogenate centrifuged at 16000*g* for 20 min at 4 °C followed by supernatant collection.

Total protein concentration was determined using a Pierce BCA Protein Assay Kit (Thermo Scientific) and a Beckman Coulter DU 730 UV/Vis Spectrophotometer (Beckman Coulter, Mississauga, Canada).

15 μg protein of each sample was diluted with one part Laemmli (loading) buffer (4% SDS, 10% 2-mercaptoethanol, 20% glycerol, 0.004% bromophenol blue, 0.125 M Tris-HCl, pH 8.0), incubated at

**Table 2**
Primer sequences used for qPCR. Product sizes are presented in base pairs and refer to expected product sizes. These are based on putative sequences from Anna's hummingbird (*Calypte anna*) (NCBI GenBank http://www.ncbi.nlm.nih.gov).

| Gene | Forward sequence | Reverse sequence | Product size |
|---|---|---|---|
| Elf1α1 | GTGTCTGTGAAAGATGTTCGCC | ATAATGACCTGTGCAGTGAAGC | 95 |
| GLUT1 | CACAGAAGGTGATTGAGGATTTCTAC | AACTGAGAAGATGGCAACAGAGAG | 116 |
| GLUT5 | TATCAGCATAGTGTGTGTCATCGTT | CACCGAAGGGATTGGACTGGC | 70 |
| KHK | TGATGGGGAGATACTGCACTCG | GAAGATGACGGCAGCGTTGAA | 94 |
| AldoB | CTCTTCTCCGCACTGTTCCTG | AGCCTCCTCTTCACTCTGACC | 74 |

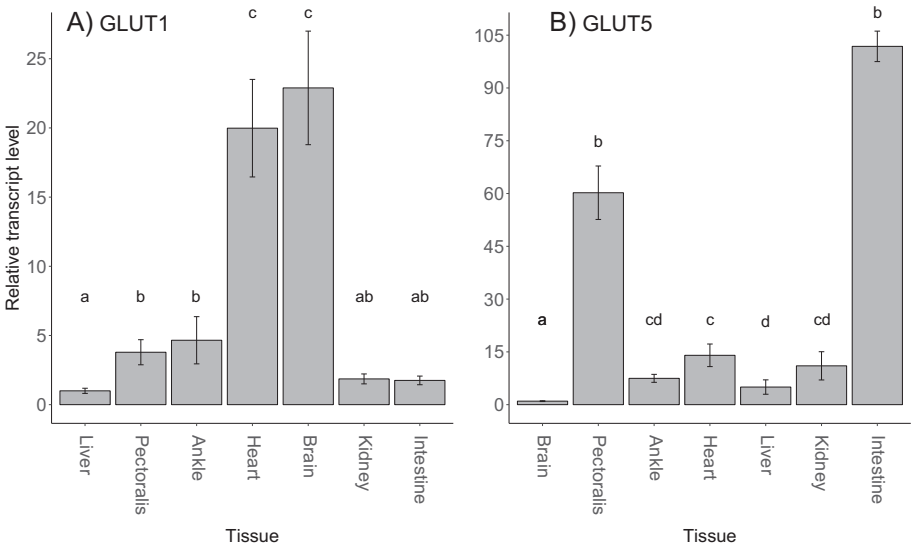


**Fig. 2.** Relative transcript abundance in ruby-throated hummingbird tissues normalized to Elf1α1 for GLUT1 (A) and GLUT5 (B). Samples are calibrated to liver tissue in (A) and to brain tissue in (B). Error bars represent standard error and tissues with different letters are significantly different ($P < 0.05$) using a linear mixed effects model with the calibrator tissue set as the intercept, with Tukey multiple comparisons; n = 6 for intestine, n = 4 for ankle musculature and kidney, and n = 7 for other tissues.

99 °C for 5 min, and separated using a Bio-Rad Mini Protean Tetra Cell (Bio-Rad) at 110 V for 100 min. 4% and 10% acrylamide stacking and separating gels were used respectively. The running buffer consisted of 192 mM glycine, 33 mM Tris, and 0.1%SDS.

Prior to transfer, the gel and nitrocellulose membrane (Invitrogen) were allowed to equilibrate in transfer buffer (192 mM glycine, 24.8 mM Tris, 1% SDS, 20% methanol) for 15 min. Protein was transferred to nitrocellulose membrane using a transfer unit (Amersham Biosciences, Little Chalfont, United Kingdom) at 70 V for 75 min. Ponceaus stain was used as a quality-control step to verify successful protein transfer. After transfer the gel was stained with 0.1% Ponceaus stain (5% acetic acid, 0.1% Ponceaus S), imaged, and then destained with deionized water.

The membrane was incubated for 1 h at room temperature 5% skim milk in TBST buffer (150 mM NaCl, 50 mM Tris, 0.1% Tween-20, pH 7.5). Anti-Aldolase B (Abcam Anti-ALDOB antibody - N-terminal ab138760 Abcam Inc., Cambridge, Massachusetts) was used at 1/100 dilution in 5% skim milk TBST and incubated for 90 min. Following incubation the membrane was washed for 5 min, five times, with TBST. The secondary antibody (Fisher Scientific Goat anti-Rabbit IgG, HRP, Polyclonal R&D Systems RDSHAF008) was then used at a 1/25000 dilution and incubated for 1 h. Following incubation the membrane was washed as before followed by a five-minute wash with TBS (150 mM NaCl, 50 mM Tris, pH 7.5). Finally, the membrane was incubated with Clarity Western ECL Blotting Substrate (Bio-Rad) for 5 min and visualized using a Bio-Rad ChemiDoc XRS + with Image Lab Software (Bio-Rad).

After visualization the membrane was stripped twice with 1 × Antibody Stripping Buffer (Gene Bio-Application L.T.D., Kfar-Hanagid, Israel) for 5 min each and washed with TBST. The membrane was blocked as above and incubated with HRP-conjugated anti-GAPDH (ab138760 Abcam) at a 1:2000 dilution in 5% milk in TBST for 1 h. The membrane was washed and visualized as described above. Bands were sized using GelAnalyzer 2010a (gelanalyzer.com). Densitometry was performed using GelQuant.NET (biochemlabsolutions.com) software. AldoB was normalized to GAPDH and tissues were calibrated to kidney. Detection of ketohexokinase protein was attempted but commercial antibodies with sufficient homology to cross-react with hummingbird protein were not available. Statistics were performed as described above but using band intensity values as input. Due to the difficulty of quantifying faint bands accurately, the band with the highest intensity was chosen as the calibrator.

### 2.7. Hexokinase activity assay

We used an assay of all HK activity to determine whether there is high capacity for rapid fructose phosphorylation by HK in ruby-throated hummingbird pectoralis. The assay was also performed with glucose substrate to verify capacity for rapid glucose phosphorylation. Samples were homogenized on ice in 50 mM imidazole-Cl homogenization buffer (1 mM EDTA, 5 mM DTT, 1% Triton X-100, pH 7.4) using a VWR VDI 112 Homogenizer (VWR, Radnor, USA) and an Omni International Sonic Ruptor 250 (Omni International, Kennesaw, USA). Enzymes and coenzymes were obtained from Sigma-Aldrich. Enzyme assays were performed at 39 °C in 50 mM imidazole-Cl assay buffer (1 mM EDTA, 5 mM glucose or fructose, 1 mM ATP, 5 mM MgCl2, 5 mM DTT, 0.5 mM NADP +, 13.5 U/ml glucose-6-phosphate-dehydrogenase (G6PDH), and 5 U/ml phosphoglucoisomerase (PGI) when fructose substrate was supplied, pH 7.4). The reactions were monitored at 340 nm in 1 ml glass cuvettes for 3 min in a Beckman Coulter DU 730 UV/Vis spectrophotometer with a Temperature Control Module (Beckman Coulter). Sugar was omitted for control runs, and prior to data collection, experiments were performed to verify that no enzymes or substrates were limiting other than endogenous HK and to ensure optimal homogenate concentration.

## 3. Results

### 3.1. GLUT1 and GLUT5 transcript abundance

GLUT1 relative transcript abundance differed significantly among tissues according to an ANOVA of the linear mixed effects model ($P < 0.0001$; Fig. 2A). Post-hoc tests showed that GLUT1 relative transcript abundance was greatest in heart and brain, and abundance was similar between these two tissues ($P > 0.99$). Abundance in heart and brain was significantly greater than in other tissues ($P < 0.001$). Average GLUT1 relative transcript abundance was next-most abundant in muscle tissues and significantly greater than that in liver ($P < 0.05$). Abundance was not significantly different between pectoralis and ankle musculature ($P = 1.000$). Abundance in muscle was not significantly greater than that in intestine and kidney ($P < 0.05$).

GLUT5 relative transcript abundance among tissues were significantly different according to an ANOVA of the linear mixed effects model ($P < 0.0001$; Fig. 2B). Post-hoc tests showed that GLUT5 relative transcript abundance was significantly greater in pectoralis and intestine than in other tissues ($P < 0.001$), and these were not significantly different from each other ($P > 0.05$). Heart had significantly

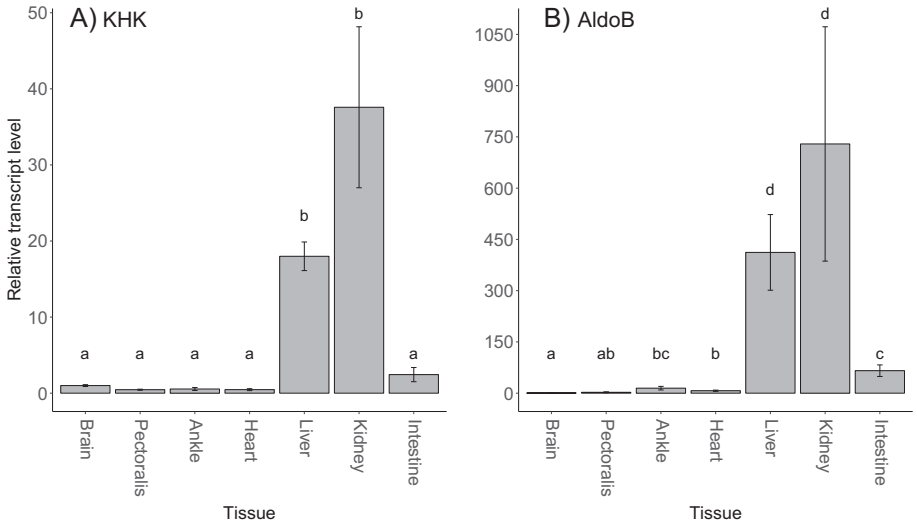


**Fig. 3.** Relative transcript abundance in ruby-throated hummingbird tissues normalized to Elf1α1 for KHK (A) and AldoB (B). Samples are calibrated to brain tissue. Error bars represent standard error and tissues with different letters are significantly different ($P < 0.05$) using a linear mixed effects model with the calibrator tissue set as the intercept, with Tukey multiple comparisons. n = 4 for ankle musculature and kidney, and n = 6 for other tissues.

greater relative transcript abundance than liver or brain ($P < 0.05$), but non-significantly different abundance from ankle musculature and kidney ($P > 0.05$). Brain had the lowest expression level ($P < 0.05$).

### 3.2. KHK and AldoB transcript abundance

KHK relative transcript abundance among tissues were significantly different according to an ANOVA of the linear mixed effects model ($P < 0.0001$; Fig. 3A). Post-hoc tests showed that relative transcript abundance was significantly higher in kidney and in liver than in other tissues ($P < 0.001$). Abundance in kidney and liver was not significantly different ($P > 0.05$). Among other tissues abundance did not differ significantly ($P > 0.05$).

AldoB relative transcript abundance differed significantly among tissues according to an ANOVA of the linear mixed effects model ($P < 0.0001$; Fig. 3B). Post-hoc tests showed that liver and kidney exhibited a significantly higher relative transcript abundance than did other tissues ($P < 0.005$), but were non-significantly different from each other ($P > 0.05$). Intestine had significantly greater relative transcript abundance than brain or heart ($P < 0.001$) and heart had significantly greater abundance than brain ($P < 0.0001$). Pectoralis did not significantly differ from brain, ankle musculature, or heart ($P > 0.05$), and ankle musculature did not differ significantly from pectoralis, heart, or intestine ($P > 0.05$).

### 3.3. Aldo-B relative protein expression

A single band was observed in all tissues near the predicted size of 39 kDa (Fig. 4), although it was very faint in most. The observed band size in hummingbird samples is consistent with the predicted size of this protein based on AldoB transcripts identified in a newly available ruby-throated hummingbird liver transcriptome (Workman et al., 2017). Consistent sampling of intestinal protein could not be confirmed so this tissue was excluded from statistical analysis. AldoB protein densities among tissues were significantly different according to an ANOVA of the linear mixed effects model ($P < 0.0001$). Post-hoc tests showed that liver and kidney protein abundance was similar ($P > 0.05$) and significantly greater than other tissues ($P < 0.001$). These results agreed with qPCR data, which had indicated low AldoB expression in muscle, heart, and brain.

### 3.4. Capacity for phosphorylation of glucose and fructose in primary flight muscle in vitro

$V_{max}$ for phosphorylation of glucose and fructose by HK were 21.09 ± 1.81 and 12.94 ± 1.70 μmol $min^{-1}$ g wet $tissue^{-1}$ ± SE, respectively, n = 7. The $V_{max}$ for fructose phosphorylation is over 50% as great as the $V_{max}$ for glucose phosphorylation.

## 4. Discussion

Several research teams have identified multiple adaptations in nectarivore cardiovascular and cellular physiology that supply blood sugar rapidly to working muscles (Beuchat and Chong, 1998; Bishop, 1997; Braun and Sweazea, 2008; Mathieu-Costello et al., 1992; Mqokeli and Downs, 2012; Vock et al., 1996). Little is known about the specializations that allow these sugars to be transported into muscle at rates sufficient to sustain hovering flight (Chen and Welch, 2014). This question is made more difficult by an ongoing lack of understanding of regulation of this transport (Braun and Sweazea, 2008). Capacity for glucose phosphorylation by hummingbird flight muscle hexokinase is calculated to be rapid enough to match the energetic demands of hovering flight *in vivo* (Chen and Welch, 2014), but capacities for hummingbird fructose phosphorylation have not been previously examined.

We observed relative transcript abundance of GLUT1 in both pectoralis and ankle musculature that exceeded that of liver (Fig. 2A). GLUT1 relative transcript abundance in muscles was comparable to both kidney and intestine. This tissue expression pattern differs from the transcript and protein tissue expression patterns of other vertebrates examined, which show relatively low GLUT1 transcript abundance in muscle relative to liver and kidney (Aschenbach et al., 2009; Bell et al., 1990; Hall et al., 2014; Sweazea and Braun, 2006; Zhao et al., 1993). Such high relative transcript abundance of GLUT1 mRNA suggests that hummingbird muscle has an elevated capacity for glucose transport into the sarcoplasm, a prerequisite for its rapid oxidation, which is supported by previous findings (Chen and Welch, 2014).

The finding that GLUT1 transcript is similarly abundant in both flight muscle and non-flight muscle is tantalizing, in that it suggests that apparent GLUT1 upregulation in hummingbird muscle is not driven purely by the need to fuel energetically expensive hovering flight. As all birds investigated lack GLUT4 (Braun and Sweazea, 2008; Carver et al., 2001; Seki et al., 2003; Sweazea and Braun, 2006; Welch et al., 2013), the most important glucose transporter in mammalian muscle for lowering blood glucose (Shepherd and Kahn, 1999), high densities of GLUT1 may be critical to facilitating rapid glucose uptake into muscle. The suggested capacity for rapid glucose uptake in the flight muscle is consistent with evidence that hummingbirds can power their flight exclusively using recently ingested (circulating) glucose. Another possibility is that the protein turnover rate of GLUT1 in hummingbird muscle is very high, perhaps as a means of regulation, and that the rate

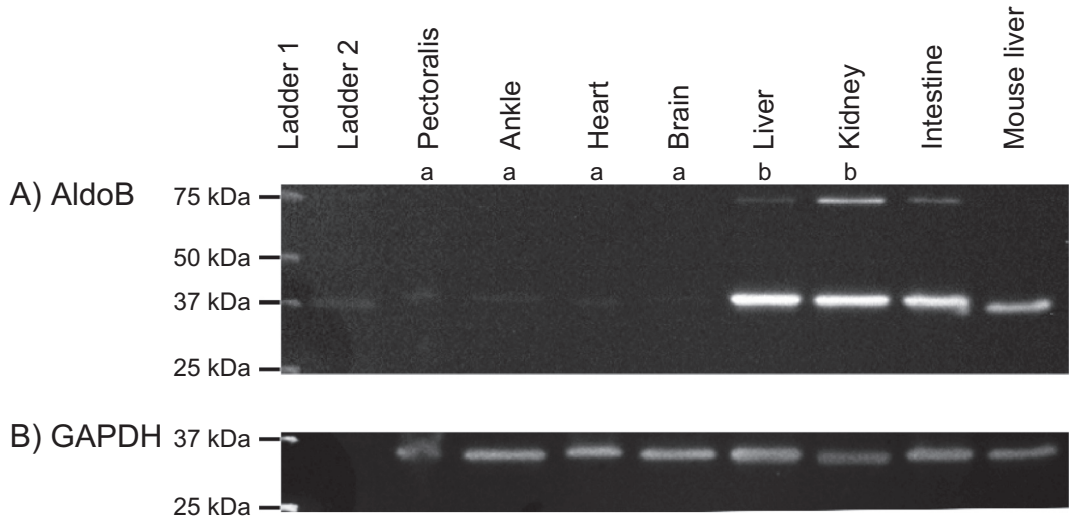


**Fig. 4.** Relative protein abundance of AldoB (A) and GAPDH (B) in ruby-throated hummingbird tissues. AldoB expression is normalized to GAPDH expression and calibrated to kidney tissue. Tissues with different letters are significantly different ($P < 0.001$) using a linear mixed effects model with kidney set as the intercept, with Tukey multiple comparisons. $n = 4$.

of transcription is similarly high. Future protein abundance studies will investigate these possibilities further. GLUT3 is another glucose transporter transcribed in hummingbird muscle (Welch et al., 2013) and expressed in vertebrate muscle in general (Thorens and Mueckler, 2010), albeit with lower abundance than GLUT1 in mammals examined (Gaster et al., 2000). GLUT3 may be similarly upregulated in abundance and presents an avenue for further study.

We observed higher GLUT5 relative transcript abundance in pectoralis than in any other tissues investigated, other than the intestine (Fig. 2B). Relative transcript abundance in heart was greater than that in liver, and abundance was similar between ankle musculature, heart, and kidney. This tissue expression pattern again differs from that observed in other vertebrates, in which the relative GLUT5 transcript and protein abundance of intestine and kidney far exceed that of skeletal muscle (Aschenbach et al., 2009; Bell et al., 1990; Rand et al., 1993; Zhao et al., 1993). Taken together with previously published respirometry and stable isotope tracking data (Chen and Welch, 2014), GLUT5 expression data suggests that the primary flight muscle of the ruby-throated hummingbird may possess relatively high capacity for fructose uptake into flight muscle. If protein quantity is similar to transcript abundance in these tissues, then it appears that step 2 of the "sugar oxidation cascade" (Suarez et al., 2011) may be comparatively less-limiting to fructose uptake and oxidation in hummingbirds than it is in other vertebrates examined thus far. This is consistent with previous findings that hummingbirds do not seem to differentiate between glucose and fructose when it comes to maintaining hovering flight and maximum metabolic activity (Chen and Welch, 2014). Future assessment of GLUT5 protein abundance and sugar uptake assays will be needed to confirm this hypothesis.

The difference between GLUT5 relative transcript abundance in flight *versus* non-flight muscle was compelling. The lack of such high GLUT5 relative transcript abundance in ankle musculature suggests that high expression in the pectoralis is related to the energetic demands of flight muscle. Lastly, greater GLUT5 relative transcript abundance in heart than in liver is in contrast to tissue expression profiles from mammalian studies (Aschenbach et al., 2009; Zhao et al., 1993), with the exception of the fructose-specialized naked mole rat (Park et al., 2017), and suggests that fructose oxidation may play a role in powering the hummingbird heart. The energetic demands of the hummingbird heart, which beats up to 1300 times a minute (Lasiewski, 1964), are likely much higher than those of the ankle musculature, which is primarily used for perching and not locomotion (Tobalske et al., 2004).

Having observed evidence that the transport capacity for fructose into flight muscle may be relatively high, we hypothesized that circulating and intracellular fructose concentrations were also relatively high. Thus, we sought to determine whether phosphorylation capacity was high enough to support proposed rapid fructose phosphorylation. We quantified the expression of rate-limiting enzymes of fructolysis to investigate the role of this pathway in proposed rapid fructose oxidation in flight muscle. Surprisingly, relative transcript abundance of KHK and AldoB in muscle was negligible (Fig. 3). KHK and AldoB relative transcript abundance was highest in liver and kidney, similar to expression patterns seen in mammalian tissue (Cirillo et al., 2009; Sun and Empie, 2012). We were able to identify a commercial antibody for AldoB that cross-reacted with hummingbird tissue. The observed band size of AldoB in hummingbird tissue matched that predicted from transcript sequences identified in the ruby-throated hummingbird liver transcriptome (Workman et al., 2017), and densitometry analysis of this rate-limiting enzyme of fructolysis (Bode et al., 1980) corroborated that there is very low relative expression of AldoB in hummingbird muscle (Fig. 4). Given previous evidence for rapid fructose oxidation in working hummingbird muscle (Chen and Welch, 2014) and low abundance of KHK and AldoB in flight muscle, we measured HK activity to investigate the possibility that fructose is rapidly phosphorylated in the glycolysis pathway, despite the very high $K_m$ for fructose measured in other vertebrate HKs (Cárdenas et al., 1998).

We measured HK activity using either glucose or fructose as the substrate. The $V_{max}$ for glucose phosphorylation by HK was 21.09 μmol $min^{-1}$ g wet $tissue^{-1}$ ± 1.81, which is in close agreement with published HK maximal activity in the closely-related rufous hummingbird (*Selasphorus rufus*; Suarez et al., 1990). Capacity for glucose phosphorylation *in vitro*, assessed as the $V_{max}$ for HK, was rapid enough to match previously calculated rates of glucose uptake by hovering muscle *in vivo* (Welch and Chen, 2014). Capacity for fructose phosphorylation, estimated the same way, was 12.94 μmol $min^{-1}$ g wet $tissue^{-1}$ ± 1.70, over 50% as great as the capacity for glucose phosphorylation. While this capacity is not great enough to match calculated rates of fructose uptake by flight muscle during hovering flight, our results did show high relative expression of KHK and AldoB in liver and kidney (Figs. 3 and 4). It seems likely that proposed capacities for rapid uptake and phosphorylation of fructose by flight muscle may be supplemented by further fructose metabolism in the liver and kidney. As in mammals (Johnson et al., 2010; Sun and Empie, 2012), these tissues may become net producers and exporters of other metabolites such as glucose, pyruvate, and lactate that are derived from fructose metabolism. Roughly half of the monosaccharide-derived

caloric intake of a hummingbird in the wild comes from glucose, with the other half coming from fructose (Baker et al., 1998). We observed evidence of capacities for rapid transport of both sugars into flight muscle as well as for their rapid subsequent phosphorylation. Taken together with the abundance of fructolytic enzymes in the liver, we hypothesize that hummingbirds have the capacity to mobilize ingested sugars to active muscle tissue and to phosphorylate and subsequently oxidize them rapidly enough to sustain rates of ATP turnover required to power the activity of hovering flight. In light of the observed capacity for fructose phosphorylation, total fructose metabolism may be sufficient to sustain some behaviours in the wild, even if fructose were the only sugar available. When calculations of fructose uptake by ruby-throated hummingbird muscle are averaged across multiple bouts of hovering and perching, which more accurately describes non-migratory, wild behaviour compared to continuous non-stop hovering, the observed capacity for fructose phosphorylation *in vitro* may be more than enough to sustain typical foraging behaviour. Chen and Welch, (2014) calculated time-averaged rates of fructose uptake by ruby-throated hummingbirds to be $6.14 \pm 0.59\ \mu mol\ min^{-1}\ g\ wet\ tissue^{-1}$, less than our calculated $V_{max}$ of HK with fructose, $12.94 \pm 1.70\ \mu mol\ min^{-1}\ g\ wet\ tissue^{-1}$.

It remains to be determined which isoforms of hexokinase are involved in proposed rapid fructose phosphorylation and what the circulating levels of blood fructose are. The abundance of fructose in the diet and the high relative transcript abundance of GLUT5 in pectoralis, suggest that blood, and likely sarcoplasmic, fructose levels are similarly high to those measured for glucose (Beuchat and Chong, 1998; Welch and Chen, 2014).

We have further characterized the adaptations that allow for apparent rapid flux of ingested sugar to catabolism in the primary flight muscle. Our findings help elucidate the strategies by which tiny endotherms maintain energy homeostasis in the face of some of the highest metabolic rates known to vertebrates (Suarez, 1992). These adaptations may be shared by other hovering nectarivores, such as nectar bats, and whether or not they constitute fundamental adaptations to hovering flight on a high fructose diet presents an avenue for future study. Finally, the recent production of the first hummingbird transcriptome (Workman et al., 2017), coupled with increasing availability of hummingbird genomic data (Zhang et al., 2014), has provided critical information for the generation of hummingbird GLUT and HK specific antibodies, as well as recombinant enzymes for functional studies.

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## Conflict of interest

The authors have no conflicts of interest to note.

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