Avian Research

RESEARCH

Open Access

No signature of selection on the C-terminal region of glucose transporter 2 with the evolution of avian nectarivory

Alexander M. Myrka[1†], Tooba Shah[2†], Jason T. Weir[2,3] and Kenneth C. Welch Jr.[2,3*]

Abstract

Background: Flying birds, especially those that hover, need to meet high energetic demands. Birds that meet this demand through nectarivory face the added challenges of maintaining homeostasis in the face of spikes in blood sugar associated with nectar meals, as well as transporting that sugar to energetically demanding tissues. Nectarivory has evolved many times in birds and we hypothesized that the challenges of this dietary strategy would exert selective pressure on key aspects of metabolic physiology. Specifically, we hypothesized we would find convergent or parallel amino acid substitutions among different nectarivorous lineages in a protein important to sensing, regulating, and transporting glucose, glucose transporter 2 (GLUT2).

Methods: Genetic sequences for GLUT2 were obtained from ten pairs of nectarivorous and non-nectarivorous sister taxa. We performed PCR amplification of the intracellular C-terminal domain of GLUT2 and adjacent protein domains due to the role of this region in determination of transport rate, substrate specificity and glucosensing.

Results: Our findings have ruled out the C-terminal regulatory region of GLUT2 as a target for selection by sugar-rich diet among avian nectarivores, though selection among hummingbirds, the oldest avian nectarivores, cannot be discounted.

Conclusion: Our results indicate future studies should examine down-stream targets of GLUT2-mediated glucosensing and insulin secretion, such as insulin receptors and their targets, as potential sites of selection by nectarivory in birds.

Keywords: GLUT2, Nectarivory, Avian, Glucosensing, Glucose, Diet, Insulin signalling

Background

To meet the high-energy demands of foraging flight, while relying on relatively dilute sugar solutions (20–25% sucrose in the case of hummingbirds; Roberts 1996), nectarivorous birds must sustain high nectar intake rates. Little is known about how nectarivorous birds are able to maintain sugar homeostasis and avoid or mitigate pathological outcomes from spikes in blood sugar concentration despite their chronic high rates of sugar intake. This metabolic challenge is exacerbated by the fact that birds generally maintain higher plasma glucose concentrations than other vertebrates of similar body mass while also storing very little glucose intracellularly as glycogen (Braun and Sweazea 2008). Most work has focused on hummingbirds and comparatively little is known about the metabolic fate of sugars in other nectarivores, but convergent evolution of dietary ecology suggests that convergent adaptations may be present (Baker et al. 1998).

*Correspondence: Kenneth.welchjr@utoronto.ca
†Alexander M. Myrka and Tooba Shah co-first authors
[2] Department of Biological Sciences, University of Toronto Scarborough, 1265 Military Trail, Toronto, ON M1C 1A4, Canada
Full list of author information is available at the end of the article

Physiological and biochemical adaptations for sugar metabolism in birds should not only require the effective regulation of plasma glucose and fructose levels, but also the capacity for rapid transportation of both sugars across the intestinal epithelium and to energetically demanding organs (e.g. flight muscles) (Shen et al. 2012). An initial avenue of investigation is to look for a signature of selection on the sequences of proteins related to glucose transport, glucosensing, and sugar homeostasis. Discovery of a signature convergent amino acid sequence associated with nectarivorous diets is a first step in identifying molecular adaptations by which animals subsist on high-sugar diets while avoiding metabolic dysfunction. Selection of a target protein for screening necessitates consideration of how avian blood glucose regulation differs from that of mammals and other vertebrates, particularly with regard to glucose transporters (GLUTs). GLUTs are key players in sugar homeostasis, able to move hydrophilic sugars across the lipid bilayer (Uldry and Thorens 2004). In addition to transporting glucose and fructose, GLUTs also play vital roles in glucosensing and blood glucose regulation, and facilitate insulin signalling (Taniguchi et al. 2006; Zhao and Keating 2007; Thorens and Mueckler 2010).

The insulin signalling pathway is not thoroughly characterized in non-mammal species, but the role of insulin appears broadly conserved in non-avian vertebrates (Polakof et al. 2011). In mammals, the insulin pathway is as follows: circulating insulin binds to extracellular insulin receptors (IRs) of target tissues, triggering phosphorylation of IR substrates, which in turn activate the phosphatidyl inositol 3′-kinase-protein kinase B (PI3K-Akt) pathway. In response, glucose transporter 4 (GLUT4)-bearing intracellular vesicles are translocated to the cell membrane (Taniguchi et al. 2006; Dupont et al. 2009) resulting in increased glucose uptake capacity (Huang and Czech 2007).

While the insulin-mediated GLUT4 response is broadly conserved among fish, reptiles, and mammals (Shepherd and Kahn 1999; Polakof et al. 2011; OsorIo-Fuentealba et al. 2013), GLUT4 is absent from the genomes of all birds examined (Carver et al. 2001; Seki et al. 2003; Sweazea and Braun 2006; Braun and Sweazea 2008). Because birds lack GLUT4, insulin does not induce uptake of blood glucose into avian muscle, heart or adipose tissues (Braun and Sweazea 2008), though birds do retain sensitivity to glucagon (Hazelwood 1973).

Despite the insensitivity of avian blood glucose to insulin and hyperactivity of PI3K in chicken muscle (Dupont et al. 2004), evidence in chickens has shown that insulin still has a regulatory role over cell metabolism in chicken hepatoma cells and primary myocytes (Duchêne et al. 2008; Dupont et al. 2009). Inhibition of chicken PI3K demonstrated that PI3K activity in both cell types is necessary for normal insulin-induced activation of both the extracellular signal-regulated kinase/mitogen-activated protein kinase (ERK/MAPK) pathways and subsequent downstream phosphorylation and activation of p70 S6 kinase (S6K1) (Duchêne et al. 2008). These pathways upregulate cell growth and protein synthesis in mammals, and likely chickens as well (Duchêne et al. 2008; Dupont et al. 2009). Together the evidence suggests that despite a lack of GLUT4, insulin regulates cellular energy budgets in liver and muscle in chickens.

It is unknown whether the phenomena impacting insulin responsiveness in chickens are at work in other avian species. With the genetic absence of GLUT4, an open question is whether a nectar diet-induced selective pressure acts on another GLUT isoform involved in sugar homeostasis in avian nectarivores, and in turn on the insulin sensitivity of avian liver and muscle. We turned our attention to the glucosensing isoform necessary for insulin and glucagon secretion in mice, GLUT2 (Wu et al. 1998; Thorens and Mueckler 2010; Long and Cheeseman 2015).

GLUT2 is most highly expressed in the gut and in key tissues of sugar homeostasis, such as intestines, liver, kidneys, and pancreatic beta islet cells of mammals, and expression is similar in Chickens (*Gallus gallus*) (Kono et al. 2005; Long and Cheeseman 2015; Byers et al. 2018). In rodent models, presence and activity of GLUT2 are required for normal glucose sensing, which precedes insulin and glucagon secretion (Thorens and Mueckler 2010), and it is one of the few GLUTs to transport both glucose and fructose, albeit with relatively low binding affinities (Wu et al. 1998; Long and Cheeseman 2015). The high mammalian K_m values of GLUT2 (17 mM for glucose and 76 mM for fructose in humans; Uldry and Thorens 2004) mean that transport capacity is not saturated at the range of serum sugar levels typically experienced in mammals. This ensures that mammalian glucose uptake rate in glucose-sensing tissues is always reactive to changes in circulating glucose (Thorens and Mueckler 2010). The mammalian protein also responds to glucose binding through interprotein interactions, leading to transcriptional regulation (Leturque et al. 2009). Broiler chickens injected with insulin demonstrated reduced liver expression of GLUT2 and IR transcripts, suggesting that GLUT2 is insulin sensitive in avian liver (Dupont et al. 2004). Decreased glucose transport capacity in liver in response to insulin signalling may serve to make glucose more available to peripheral tissues (Zhang et al. 2013). Caution must be taken in interpreting the results of Zhang et al. (2013), as much greater than physiologically relevant levels of insulin were administered to achieve a response. Still, the capacity for

insulin-stimulated GLUT2 regulation was nonetheless demonstrated.

Recently it was demonstrated that GLUT2 protein is constitutively expressed in both liver and primary flight muscle of the Ruby-throated Hummingbird (*Archilochus colubris*); further, dietary status altered GLUT2 protein expression in whole tissue homogenates of flight muscle but not liver (Ali et al. 2020). No change in plasma membrane recruitment of GLUT2 was seen in hummingbird tissues between fed and fasted states. Whole tissue GLUT2 was, however, greater in the flight muscle of fed hummingbirds than in fasted ones, which may indicate regulation of a recruitable GLUT2 population or, alternatively, regulation of GLUT2 protein turnover (Ali et al. 2020). A lack of change in observed plasma membrane GLUT2 suggested that the primary role of this transporter is not in direct modulation of sugar uptake rate, but likely in glucosensing and regulation as described in other systems (Thorens 1996; Thorens and Mueckler 2010; Ali et al. 2020). As avian nectarivores, including hummingbirds, share the metabolic challenges of feeding on sugar-rich nectar (Baker et al. 1998), we predicted that GLUT2 would have a similar and convergent role in glucosensing and sugar homeostasis in other avian nectarivores.

Given evidence of selection on GLUT4 in relation to dietary ecology and nectarivory in bats (Shen et al. 2012), but that birds lack GLUT4 and appear to utilize GLUT2 in insulin responsiveness and glucose homeostasis (Dupont et al. 2004; Ali et al. 2020), we hypothesized that we would find a signature of nectarivorous dietary ecology in the protein sequence of avian GLUT2.

Amino acid sequence divergence may be slow to accumulate in highly-conserved regions where mutation is likely to disrupt protein functions. We therefore narrowed our investigation to a region of the GLUT2 protein where sequence divergence was high. Sequence alignments reveal high conservation in the interior of the protein, while the N and C termini are the most highly divergent sections of the protein, in both sequence and length, among isoforms and orthologs (Katagiri et al. 1992; Zhao and Keating 2007). Amino acid sequences in transmembrane segments 9–12 of GLUT2 influence substrate affinity for both glucose and fructose (Wu et al. 1998). The C-terminal domain of GLUTs is required for the transport of glucose, yet it is diverse in size and amino acid sequence among GLUT isoforms and orthologs (Katagiri et al. 1992; Zhao and Keating 2007). It also plays a role in determining the rate of glucose transport across membranes (Katagiri et al. 1992) and the C-terminal broadly contributes to determining the kinetic properties unique to each GLUT isoform (Katagiri et al. 1992). For these reasons, we focused our study on the C-terminal region and adjacent primary protein sequence. We hypothesized that there was sufficient flexibility of the primary amino acid sequence for mutations to accumulate relatively rapidly in evolutionary time, permitting evolution of a signature of nectarivorous diet.

To conduct our study, we considered as many instances of the evolution of avian nectarivory, through paired sister contrasts, as obtainable samples permitted. We were able to obtain tissue samples from 28 species representing ten paired contrasts (Fig. 1, Table 1). Not much is known about glucose metabolism and sugar homeostasis in most of the included species, but by utilizing paired sister contrasts of greatly varied morphology and evolutionary history, we aimed to gain insight into sugar homeostasis applicable to all avian nectarivores. We performed PCR amplification and Sanger sequencing from exon 10–11 of the gene, which encode from the end of TM9 through most of the C-terminal domain (Katagiri et al. 1992). With the differing foraging behaviours of nectarivores and non-nectarivores, we predicted that we would observe a convergent signature for nectarivory in the amino acid sequence of the C-terminal end of GLUT2 among clades representing independent origins of nectarivory.

Methods

Species coverage and primer design

Diet was categorized using *The Handbook of the Birds of the World* (Irestedt and Ohlson 2008; Schuchmann 2015) and species in which nectar eating was documented were noted, 1095 species in all (Additional file 1: Table S1). After identifying nectar-consuming species, we used phylogenetic trees from the literature to perform ancestor state reconstruction to identify discrete instances of the evolution of nectarivory (Burns et al. 2003; Warren et al. 2006; Zhang et al. 2007; Hackett et al. 2008; Irestedt and Ohlson 2008; Wright et al. 2008; Hedges and Kumar 2009; Reding et al. 2009; Weir et al. 2009; Jønsson et al. 2010; Sedano and Burns 2010; Moyle et al. 2011). Multiple independent instances of the evolution of nectarivory, as well as nearest non-nectarivorous taxa for each instance of the evolution of nectarivory, were identified (Additional file 1: Table S2). Pectoralis muscle tissue samples of target species were obtained from either the Field Museum of Natural History (Chicago, Illinois, USA) or from the collections of the Weir and Welch laboratories (University of Toronto, Canada). We acquired 28 samples representing ten of the 22 identified clade pairs of unique origins of nectarivory (Table 1; Additional file 1: Table S2), and for each we performed Sanger sequencing of PCR products of the C-terminal end of the SLC2A2 gene (encoding GLUT2) for one or a few nectarivorous species from each clade and the same for each of their

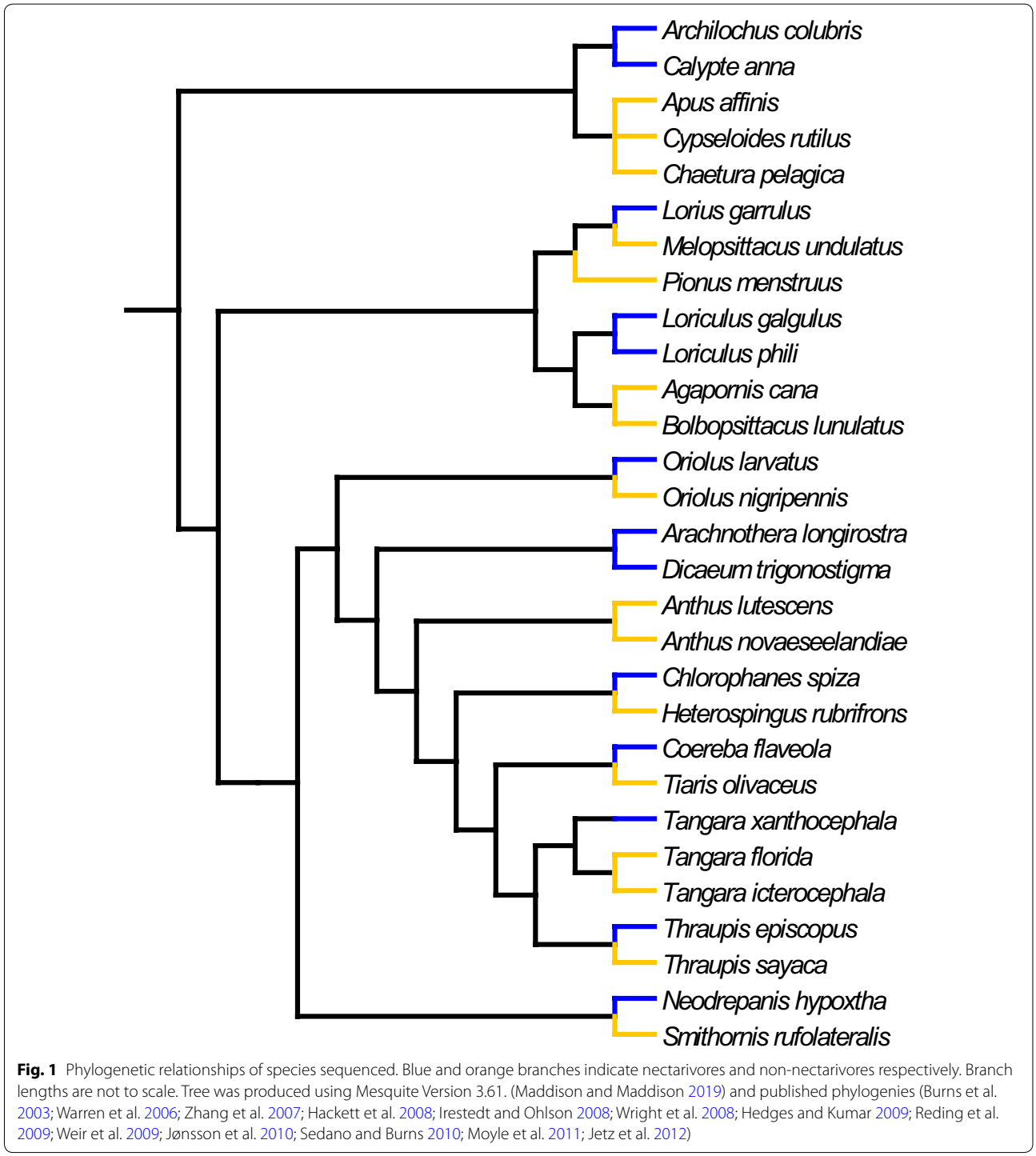

Fig. 1 Phylogenetic relationships of species sequenced. Blue and orange branches indicate nectarivores and non-nectarivores respectively. Branch lengths are not to scale. Tree was produced using Mesquite Version 3.61. (Maddison and Maddison 2019) and published phylogenies (Burns et al. 2003; Warren et al. 2006; Zhang et al. 2007; Hackett et al. 2008; Irestedt and Ohlson 2008; Wright et al. 2008; Hedges and Kumar 2009; Reding et al. 2009; Weir et al. 2009; Jønsson et al. 2010; Sedano and Burns 2010; Moyle et al. 2011; Jetz et al. 2012)

non-nectarivorous contrasts. Relationships of species sequenced are shown in Fig. 1 and explained in detail in supplementary materials (Additional file 2; Additional file 3: Fig. S1–S4).

In order to identify conserved sequences of the C-terminal end of the avian SLC2A2 gene for primer design, exons 10 and 11, as well as the encompassed intron, of 47 randomly chosen birds were obtained from NCBI GenBank (Additional file 4: Table S3; Benson et al. 2005) and

Table 1 Representative species from divergent taxa of nectarivorous (with double asterisks) and non-nectarivorous (without asterisks) diet used in this study

Contrast	Clades compared	Sequenced representatives of each contrast	Common name
1	Lorikeets and budgerigars and an outgroup	*Lorius garrulus* (nectarivore)**	Chattering Lorry
		Melopsittacus undulatus (non-nectarivore)	Budgerigar
		Pionus menstruus (non-nectarivore)	Blue-headed Parrot
2	Sunbird-asities and broadbills	*Neodrepanis hypoxtha* (nectarivore)**	Yellow-bellied Sunbird-asity
		Smithornis rufolateralis (non-nectarivore)	Rufous-sided Broadbill
3	Hanging parrots and lovebirds/guaiaberos	*Loriculus galgulus* (nectarivore)**	Blue-crowned Hanging Parrot
		Loriculus phili (nectarivore)**	Philippine Hanging Parrot
		Agapornis cana (non-nectarivore)	Grey-headed Lovebird
		Bolbopsittacus lunulatus (non-nectarivore)	Guaiabero
4	Hummingbirds and swifts	*Calypte anna* (nectarivore)**	Anna's Hummingbird
		Archilochus colubris (nectarivore)**	Ruby-throated Hummingbird
		Apus affinis (non-nectarivore)	House Swift
		Cypseloides rutilus (non-nectarivore)	Chestnut-collared Swift
		Chaetura pelagica (non-nectarivore)	Chimney Swift
5	Saffron-crowned tanager and related tanagers	*Tangara xanthocephala* (nectarivore)**	Saffron-crowned Tanager
		Tangara florida (non-nectarivore)	Emerald Tanager
		Tangara icterocephala (non-nectarivore)	Silver-throated Tanager
6	Flowerpeckers/sunbirds and motacillidae	*Arachnothera longirostra* (nectarivore)**	Little Spiderhunter
		Dicaeum trigonostigma (nectarivore)**	Orange-bellied Flowerpecker
		Anthus lutescens (non-nectarivore)	Yellowish Pipit
		Anthus novaeseelandiae (non-nectarivore)	Newzealand Pipit
7	Green Honeycreeper and related tanagers	*Chlorophanes spiza* (nectarivore)**	Green Honeycreeper
		Heterospingus rubrifrons (non-nectarivore)	Sulphur-rumped Tanager
8	*O. larvatus* and *O. nigripennis*	*Oriolus larvatus* (non-nectarivore)**	Black-headed Oriole
		Oriolus nigripennis (nectarivore)	Black-winged Oriole
9	*T. episcopus* and *T. sayaca*	*Thraupis episcopus* (nectarivore)**	Blue-gray Tanager
		Thraupis sayaca (non-nectarivore)	Sayaca Tanager
10	Banaquits and grassquits	*Coereba flaveola* (nectarivore)**	Bananquit
		Tiaris olivaceus (non-nectarivore)	Yellow-faced Grassquit

Arbitrary numbers are assigned to each contrast and dietary category is indicated

aligned using MEGA 5.2 Alignment software (Tamura et al. 2011). Because our pairwise comparisons spanned widely across the avian lineage, the alignment of randomized birds from across the Aves taxa allowed us to identify small, conserved target sites for primer design, such that the same primer sets could be used for all samples.

The most conserved regions of exon 10 and exon 11 in the alignment of the SLC2A2 gene were identified and primers were designed for these two exons to ensure amplification of samples from all species. Custom primers (value oligos) were synthesized by Thermo Fisher Scientific (Waltham MA, USA). The primer set for exon 11 (E11) was designed to span the intron between exons 10 and 11 and to be overlapping with the primer set for exon 10 (E10). The 5′ to 3′ sequences for E10 were GAGCTATGTCAGCATGGTCG (forward) and AGG GAATTCTTACCGCTATGTACTG (reverse). Annealing temperature was 54.5 °C and predicted product length was 200 base pairs. The respective sequences for E11 were GAATGTGTTTCCAGTACATAGCGG (forward) and GCTGGATGCTTCTTGCGGC (reverse). Annealing temperature was 55.5 °C and predicted average product length was 334 base pairs. Because E11 spanned an intron, the actual predicted product size varied slightly due to intron variability. The average predicted size is based on sequences listed in Additional file 4: Table S3. The primer sets were checked for specificity using NCBI Primer Blast (Ye et al. 2012) and were tested using mfold (Zuker 2003) to check for significant secondary structures in the template that might hinder primer binding. Both primer sets were tested on three different bird species that are distantly related (*Apus affinis subfurcatus* (Swifts), *Agapornis*

cana (Grey-headed Parrot), and *Arachnothera longirostra* (Little Spider Hunter)), to verify specificity.

DNA isolation

DNA extraction was performed using the E.Z.N.A tissue DNA kit (Omega Bio-tek Inc., Georgia, USA) following the manufacturer's instructions with the following modifications: due to limited tissue quantity, masses used were less than that recommended, all the centrifuging times were increased by 30 s, and DNA was eluted in 50 µL of sterile water rather than 100 µL of elution buffer. DNA purity was assessed by 260 nm/230 nm UV absorbance with a NanoDrop 1000 (Thermo Fisher Scientific). DNA integrity was verified by 1% agarose gel electrophoresis.

PCR and sequencing

20 µL reactions were performed using 0.5 µM of forward and reverse primers, 1× colorless GoTaq DNA polymerase buffer, 2 mM Magnesium Chloride, 200 µM dNTPs, 1 U/µL GoTaq (Promega Corporation, Madison WI, USA), and approximately 20–40 ng/µL of genomic DNA depending on yield of DNA extraction. Reactions were performed in an Applied Biosystems Thermocycler (Model#9902) using 2 min at 95 °C followed by 30 cycles of 95 °C for 1 min, primer-specific annealing temperature for 1 min, 72 °C for 1 min, and a final extension step of 72 °C for 5 min. The annealing temperatures were 54.5 °C and 55.5 °C for primer sets E10 and E11 respectively. For reactions with low product yield the number of PCR cycles was increased to 35. In the event of non-specific amplification, template and magnesium concentrations were adjusted incrementally until specific amplification was achieved. Amplification specificity was verified using 1% agarose gel electrophoresis.

The PCR product was then cleaned with ExoSap-IT (ThermoFisher Scientific, US) following the manufacturer's protocol and using the same thermocycler described above. Products were sent to The Center for Applied Genomics (TCAG) (MacDonald et al. 2014) for Sanger sequencing using the "difficult template" reaction chemistry option.

Data analysis

Sequences were aligned using the Mega 5.2 alignment software after translating the sequences using Expasy server (Gasteiger et al. 2003; Additional file 4: Table S4).

The amino acid sequences for *Calypte anna* (Anna's Hummingbird; nectarivore; Accession#XM_008501315.1) and *Chaetura pelagica* (Chimney Swift; non-nectarivore; Accession #XM_010006840.1) were obtained from NCBI GenBank (Benson et al. 2005) to supplement our data and to use as reference points for aligning avian nectarivore and non-nectarivore GLUT2. The downloaded swift sequence was compared to our sequence from the same species in order to verify accuracy of sequencing. Subsequent to the start of our analyses, a de novo transcriptome from the Ruby-throated Hummingbird (Workman et al. 2018) was published, allowing us to verify the Ruby-throated Hummingbird sequence that we produced. We identified amino acid differences between nectarivorous and non-nectarivorous birds in each contrast in the protein alignments and, depending on the number of representative species sequenced per contrast, performed either 2×2 or 3×2 Fisher's Exact Tests using R ver. 3.3.3 (R Core Team 2020) to determine the statistical significance of these substitutions as putative signatures for nectarivory (Additional file 4: Table S5). A p-value < 0.05 was taken to be significant.

Results

Confirmation of sequence identity and subsequent sequence alignment

GLUT2 protein sequences encoded by exons 10 and 11 in 10 paired contrasts of nectarivorous and non-nectarivorous avian taxa were sequenced and these were translated into putative protein sequences (Additional file 4: Table S4). Primer set E10 showed amplification of the products at the expected product size with no non-specific amplification. Primer set E11 showed amplification of the products at or within a few dozen base pairs of the average expected product size with no non-specific amplification. Correct sequence amplification was confirmed through manual examination of chromatograms and resulting sequences. Our generated Chimney Swift sequence was identical to that downloaded from NCBI GenBank, and our generated Ruby-throated Hummingbird sequence was identical to that generated by Workman et al. (2018), further supporting accuracy of our Sanger sequencing. Both examined exons of GLUT2 were well conserved with only a few amino acid substitutions among avian species examined. Amino acid sequence variation was observed between nectarivores and non-nectarivores within contrasts 3, 4, 6, and 8 (Table 2). None of these substitutions correlated with diet across all ten contrasts (Table 2).

Amino acid variation in exon 10 was only observed in contrast 4 at AA 411 and AA 447

Two substitutions were found when comparing exon 10 of contrast 4, which contrasted nectarivorous hummingbirds with non-nectarivorous swifts (Table 2). Valine (Val) was replaced by Isoleucine (Ile) at position 411 in hummingbirds, and Val was replaced by Threonine (Thr) at position 447 in hummingbirds, near the end of exon 10. All other birds examined had Valine at these positions

Table 2 Amino acid variation in sequences examined

Contrast number	Exon 10		Exon 11				
	411	447	476	480	484	486	490
1	V	V	V	A	V	F	Y
	V	V	V	A	V	F	Y
2	V	V	A	A	L	F	Y
	V	V	A	A	L	F	Y
3	V	V	A/V	A/G	V/I	F	Y/H
	V	V	A/V	A/G	V/I	F	Y
4	I	T	V	A	I	F	Y
	V	V	V	A	V/I	L/F	Y/H
5	V	V	A	G	I	F	H
	V	V	A	G	I	F	H
6	V	V	A	G	I	F	Y
	V	V	A	G	I	F	H
7	V	V	A	G	I	F	H
	V	V	A	G	I	F	H
8	V	V	A	A	I	F	Y
	V	V	A	G	I	F	Y
9	V	V	A	G	I	F	H
	V	V	A	G	I	F	H
10	V	V	A	G	I	F	H
	V	V	A	G	I	F	H

Nectarivores are indicated by grey shadowing and their non-nectivarous contrasts by white. Variation between hummingbirds and swifts (contrast 4) is highlighted

(Table 2). As this was only one of ten contrasts, we did not detect a statistically significant signature of convergent selection on exon 10 of GLUT2 associated with the evolution of nectarivory ($p > 0.05$; Additional file 4: Table S5).

Amino acid variation in exon 11 was observed in five positions

In exon 11, 5 variable amino acid identities were observed among numbered paired contrasts. Table 2 and Additional file 4: Table S5 show the substitutions found at each location in each of the ten contrasts. Substitutions were observed at positions 476, 480, 484, 486, and 490 (Table 2). At all locations except for 486, amino acid identity varied among numbered contrasts.

Variation at four amino acid positions in contrast 3

Variation at amino acid position 476, 480, 484, and 490 was observed among species in contrast 3 (Hanging parrots and Lovebirds/Guaiaberos; Table 2), but no amino acid identity differed consistently between the nectarivorous and non-nectarivorous categories. Rather, both amino acids were found in the nectarivorous and/or the non-nectarivous category, so the variation could not be said to correlate with diet ($p > 0.05$; Additional file 4: Table S5).

Variation in AA 480 in contrast 8

At position 480, a difference was seen with diet in contrast 8 (Black-headed Oriole and Black-winged Oriole; Table 2) with the nectarivore having alanine (Ala) and the non-nectarivore having Glycine (Gly). Among other contrasts, both Ala and Gly were observed in nectarivores and non-nectarivores, and so this variation could not be correlated with nectar diet among birds ($p > 0.05$; Additional file 4: Table S5).

Variation in AA 486 in contrast 4

All birds sequenced had Phenylalanine (Phe) at position 486, with the exception of two out of three swifts sequenced, which had Leucine (Leu; Table 2; Additional file 4: Table S4). This variation was not indicative of avian diet ($p > 0.05$; Additional file 4: Table S5).

Variation at AA 490 in contrast 6

At position 490 variation was observed within contrast 6 (Flowerpeckers/Sunbirds and Motacillidae; Table 2). Within contrast 6, the nectarivore had the amino acid Tyrosine (Tyr), while the non-nectarivore had Histidine (His); however, because both Tyr and His were observed among both nectarivores and non-nectarivores in this study, the variation within amino acid 490 cannot be attributed to diet with statistical significance ($p > 0.05$; Additional file 4: Table S5).

Variation at AA 484 and 490 in contrast 4

Variation was also seen within contrast 4 at positions 484 and 490, but this variation did not correlate with diet within contrast 4 or among birds (Table 2; $p > 0.05$; Additional file 4: Table S5).

No AA identity correlated with diet across multiple contrasts

In summary, when all ten contrasts were considered using Fisher's Exact Tests (Additional file 4: Table S5), no amino acid substitution exhibited a significant signature of convergence in nectarivores across all contrasts ($p > 0.05$; Additional file 4: Table S5).

Discussion

We tested for a signature of convergent selection on the primary amino acid sequence of GLUT2 associated with the evolution of a sugar-rich nectarivore diet. We did not detect a signature of nectarivory reflected in GLUT2 sequence divergence across the contrasts included in this study and protein sequences were mostly conserved among avian taxa. While we did identify two amino acid substitutions in hummingbirds relative to not just swifts, but all other birds examined, these substitutions were only observed in one contrast; thus, they could not be concluded to be adaptive to nectarivory among birds. No evidence was found for convergent evolution within the functional gene of interest among avian nectarivores, and this conclusion is strengthened by our consideration of ten phylogenies representing ten different instances of macroevolution of nectarivory. Hummingbirds are the oldest known avian nectarivores dating back to 33 million years ago and show the most specialization for nectar feeding amongst avian nectarivores (Nicolson and Fleming 2003). As such, there has been ample time for natural selection due to nectarivory to act on these birds and it is not improbable for the two substitutions found only in hummingbirds to be functionally relevant.

It is surprising not to find evidence of convergent selection on the regulatory (Katagiri et al. 1992) C-terminal region of GLUT2 in nectarivores, given the large variation in blood sugar levels that examined specialist nectarivores experience (17–42 mM glucose in hummingbirds; Beuchat and Chong 1998; 11.52–16.51 mM in amethyst sunbirds; Witteveen et al. 2014), as well as GLUT2′s posited role in avian insulin signaling and glucosensing (Zhang et al. 2013; Ali et al. 2020). In interpreting the implications of no signature of selection we first reconsider the state of knowledge of avian GLUT2 in glucose metabolism.

The GLUT isoform responsible for pancreatic gluocosensing may vary among animals (Mueckler and Thorens 2013), but GLUT2 is the most likely candidate in birds, with transcript expression orders of magnitude higher than that of other GLUT isoforms in chicken pancreas (Byers et al. 2018). Furthermore, liver GLUT2 transcript expression in chickens (Kono et al. 2005; Byers et al. 2018), and protein expression in hummingbirds (Ali et al. 2020), are the most abundant of GLUT isoforms investigated, further suggesting a role for GLUT2 in avian glucosensing. There is evidence that the response of GLUT2 to dietary status may vary among birds, which leads to a prediction of selection upon GLUT2 by diet. GLUT2-mediated glucosensing in mammalian liver is implicated in control of insulin secretion through a liver-beta islet axis (Thorens 2015). While Zhang et al. (2013) found preliminary evidence that chicken GLUT2 may have the capacity to respond to insulin in liver through modulation of transcription rate, Ali et al. (2020) found evidence that hummingbird GLUT2 protein degradation rate may respond to dietary status in flight muscle but not liver, so uniform responsiveness of GLUT2 to dietary status among birds is uncertain.

The importance of GLUT2 in avian nectarivores may extend to skeletal muscle more so than is the case in mammals. The constitutive presence of GLUT2 in hummingbird whole liver, and decrease of GLUT2 in hummingbird whole flight muscle with unchanging abundance in flight muscle plasma membrane fraction, suggests that GLUT2 may play a role in glucosensing, and perhaps in modulation of glucose uptake capacity, in flight muscle (Ali et al. 2020). This is especially relevant given that GLUT2 is absent in mammalian muscle (Gaster et al. 2000). In avian taxa, in which examined muscle lacks GLUT4 and IRs appear broadly insensitive to insulin with regard to induction of PI3K expression, though still necessary for downstream signal transduction (Duchêne et al. 2008; Dupont et al. 2009), GLUT2 may act as a glucosenser independently of activity level of the PI3K-Akt pathway.

In light of the aforementioned evidence that GLUT2 is key to avian glucosensing and insulin regulation, but also no detected signature of selection by diet, it is necessary to consider the possibility of selection downstream of sugar uptake by tissues. In muscle, uncharacterized

transcription factors or uncharacterized GLUT2 receptors, rather than GLUT2 itself, may contain a signature of nectarivory such as we hypothesized that we would detect in GLUT2 (Leturque et al. 2009).

Chicken liver IR and GLUT2 transcripts have the capacity to respond to insulin (Dupont et al. 2004; Zhang et al. 2013). In hummingbird muscle, fasting reduced whole tissue GLUT2 protein with unknown downstream consequences (Ali et al. 2020). Taken together these results implicate IRs or downstream messengers as possible targets of convergent selection in the livers of nectarivores, following GLUT2-mediated glucosensing as well as GLUT2-mediated insulin secretion. GLUT2 was a promising protein in which to look for a signature of convergent selection, but if it was ruled out, IRs and members of the PI3K-Akt pathway would become tantalizing targets for future investigations.

Another major player in glucosensing is Hexokinase 4 (glucokinase; GK). It is expressed primarily in the liver and pancreas of mammals (Rideau et al. 2010) and has been detected in the livers of chicken and quail (Wals and Katz 1981). GK phosphorylates glucose transported into the cell by GLUT2 and other transporters (Polakof et al. 2011). The rate of glucose phosphorylation by glucokinase transmits information on the rate of glucose uptake by GLUT2, which reflects blood glucose concentration (Polakof et al. 2011). Changes in the glucokinase gene, rather than GLUT2, may confer variation in function that modulate glucosensing and sugar homeostasis in nectarivorous birds and will be a target of future study.

Lack of an observed signature could be indictive of novel GLUT function or kinetics among birds, relative to mammals. In order to serve an effective glucosensing function GLUT2-mediated glucose uptake rate in nectarivores must be responsive to an elevated range of glucose concentration compared to non-nectarivores (Blem 1976; Braun and Sweazea 2008). For this reason, one would expect nectarivore GLUT2 to have a higher K_m than that of non-nectar eating birds, in order to prevent saturation of the transporter during rises in blood glucose following feeding. The C-terminal end of the protein is influential in determining substrate affinity and transporter kinetics (Katagiri et al. 1992) but, despite this, we did not find differences in amino acid sequence among diets. This could suggest that, regardless of avian species or dietary ecology, the kinetics of avian GLUT2 are such that GLUT2 is not saturated to maximal transport velocity, even at the high blood glucose concentrations of nectarivores, including hummingbirds (Blem 1976; Braun and Sweazea 2008). If this was the case, avian GLUT2 might be preadapted to handle high concentrations of circulating sugar, as compared to model mammalian organisms. Alternatively, other areas of the protein, such as the substrate binding site, may differ between nectarivores and non-nectarivores. Another potential explanation is that GLUT2-mediated transport indeed reaches V_{max} in the glucosensing tissues of some birds. If so, it would be unknown by what mechanism these birds sense differences in blood glucose concentrations in high ranges.

Existing literature led us to suspect that the region of GLUT2 examined in this study was the most promising target in which to look for a signature of diet and so we focused our efforts there, but other regions of the protein, such as transmembrane segments 7–8 (Wu et al. 1998) and the QLS motif (Seatter et al. 1998), also exert influence on substrate specificity and kinetics and may vary among species and diets. Future cloning and transport kinetics studies will determine the K_m of hummingbird GLUT2, following recent hummingbird transcriptome sequencing (Workman et al. 2018). Another target for future investigations is the large intracellular loop which, like the C terminal, is involved in signal transduction (Guillemain et al. 2000).

Conclusions

Although we predicted that we would see convergent mutations across nectarivorous taxa relative to non-nectarivorous contrasts, we did not find a robust signature for convergence in GLUT2 sequences amongst birds with independent origins of nectarivory. We did, however, identify two amino acid substitutions unique to hummingbird GLUT2, which present targets for future functional investigation. Our results advance the state of knowledge of glucosensing in avian nectarivores by ruling out the regulatory C-terminal end and adjacent region of GLUT2 as a site of convergent selection by nectarivory (with the possible exception of hummingbirds). This finding allows future studies to home in on targets downstream or parallel to GLUT2 in the glucosensing pathways. GK, IRs, and downstream members of the PI3K-Akt pathway should be investigated in follow-up studies. Future genome-wide comparisons can be leveraged to screen these pathways for signatures of diet and expand upon this initial study.

Supplementary information

Supplementary information accompanies this paper at https://doi.org/10.1186/s40657-020-00231-8.

Additional file 1: Table S1. List of bird species in which nectarivory has been noted in *The Handbook of the Birds of the World* (Irestedt and Ohlson 2008; Schuchmann 2015). **Table S2.** Nectarivore and non-nectariovre contrasts identified through ancestor state reconstruction of nectarivores represented in Table S1.

Additional file 2. Description of sister contrasts used in this study.

Additional file 3: Fig. S1. Ancestor state reconstruction of nectarivorous diet among lorikeets, budgerigars, and nearest related taxa. **Fig. S2.** Ancestor state reconstruction of nectarivorous diet among the Saffron-crowned Tanager and a sister taxon of non-nectarivorous tanagers. **Fig. S3.** Ancestor state reconstruction of nectarivorous diet among the sunbirds and flowerpeckers and related taxa. **Fig. S4.** Ancestor state reconstruction of nectarivorous diet among Green Honeycreeper, Golden-collared Honeycreeper, and a sister taxon of non-nectarivorous tanagers.

Additional file 4: Table S3. GenBank SLC2A2 sequences used for multiple alignment for primer design. **Table S4.** GLUT2 protein sequences encoded by exons 10 and 11 in 10 paired contrasts of nectarivorous and non-nectarivorous birds. **Table S5.** 2 × 2 and one 3 × 2 Fisher's Exact Test on the substitutions found in exons 10 and 11 in ten contrasts.

Acknowledgements
We thank The Field Museum of Natural History (Chicago, USA) for loaning tissues to us for this project and Mark Peck at the Royal Ontario Museum (Toronto, CAN) for assistance with importing tissue samples to Canada. We also thank undergraduate research students Supan Parikh, Bishoy Lawendy and Elizabeth Orguntuwase for assistance with sample processing and preliminary data collection. A special thank is given to Raafay Syed Ali for critical revision of the manuscript in the context of recent glucosensing literature. Lastly, we thank Dr. Derrick Groom, Dr. Brandy Velten, Lily Hou, and Dr. Aarthi Ashok for technical support and advice.

Authors' contributions
Literature review for categorization of avian diet was performed by AMM. AMM and TS made equal contributions to the manuscript. Both AMM and TS isolated DNA, designed and tested primers, performed PCR and sample preparation for sequencing, analyzed the data, and prepared the manuscript. JTW performed ancestor state reconstruction to identify sister contrasts, designed the statistical analysis, and provided editorial contributions to the manuscript. KCW conceived and supervised the study and provided editorial contributions to the manuscript. All authors read and approved the final manuscript.

Funding
This work was supported by grants from Natural Sciences and Engineering Research Council of Canada Discovery Grant (Number 386466 to KCW and 06538 to JTW) and the Human Frontier Science Program (Grant Number RGP0062/2016).

Availability of data and materials
All data generated or analysed during this study are included in this published article and its supplementary information files.

Ethics approval and consent to participate
All applicable international, national, and/or institutional guidelines for the care and use of animals were followed. All specimens included in this study were obtained from banked tissue at the Field Museum of Natural History (Chicago, USA) or were obtained as part of other research projects at the University of Toronto Scarborough, published elsewhere.

Competing interests
The authors declare that they have no competing interests.

Author details
[1] Department of Cell and Systems Biology, University of Toronto, 25 Harbord St., Toronto, ON M5S 3G5, Canada. [2] Department of Biological Sciences, University of Toronto Scarborough, 1265 Military Trail, Toronto, ON M1C 1A4, Canada. [3] Department of Ecology and Evolutionary Biology, University of Toronto Scarborough, 1265 Military Trail, Toronto, ON M1C 1A4, Canada.

Received: 17 April 2020 Accepted: 28 October 2020
Published online: 06 November 2020

References

Ali RS, Morag FD, Muhammad S, Sarver D, Hou L, Wong GW, et al. Glucose transporter expression and regulation following a fast in the ruby-throated hummingbird *Archilochus colubris*. J Exp Biol. 2020;22:9989. https://doi.org/10.1242/jeb.229989.
Baker HG, Baker I, Hodges SA. Sugar composition of nectars and fruits consumed by birds and bats in the tropics and subtropics. Biotropica. 1998;30:559–86.
Benson DA, Karsch-Mizrachi I, Lipman DJ, Ostell J, Wheeler DL. GenBank. Nucleic Acids Res. 2005;33:D34–8.
Beuchat CA, Chong CR. Hyperglycemia in hummingbirds and its consequences for hemoglobin glycation. Comp Biochem Physiol A Mol Integr Physiol. 1998;120:409–16.
Blem CR. Patterns of lipid storage and utilization in birds. Integr Comp Biol. 1976;16:671–84.
Braun EJ, Sweazea KL. Glucose regulation in birds. Comp Biochem Physiol B Biochem Mol Biol. 2008;151:1–9.
Burns KJ, Hackett SJ, Klein NK. Phylogenetic relationships of Neotropical honeycreepers and the evolution of feeding morphology. J Avian Biol. 2003;34:360–70.
Byers M, Bohannon-Stewart A, Khwatenge C, Alqureish C, Alhathlol A, Nahashon S, et al. Absolute quantification of tissue specific expression of glucose transporters in chickens. J Mol Cell Biol. 2018;1:1–8.
Carver FM, Shibley IA Jr, Pennington JS, Pennington SN. Differential expression of glucose transporters during chick embryogenesis. Cell Mol Life Sci CMLS. 2001;58:645–52.
Duchêne S, Audouin E, Crochet S, Duclos MJ, Dupont J, Tesseraud S. Involvement of the ERK1/2 MAPK pathway in insulin-induced S6K1 activation in avian cells. Domest Anim Endocrinol. 2008;34:63–73.
Dupont J, Dagou C, Derouet M, Simon J, Taouis M. Early steps of insulin receptor signaling in chicken and rat: apparent refractoriness in chicken muscle. Domest Anim Endocrinol. 2004;26:127–42.
Dupont J, Tesseraud S, Simon J. Insulin signaling in chicken liver and muscle. Gen Comp Endocrinol. 2009;163:52–7.
Gasteiger E, Gattiker A, Hoogland C, Ivanyi I, Appel RD, Bairoch A. ExPASy: the proteomics server for in-depth protein knowledge and analysis. Nucleic Acids Res. 2003;31:3784–8.
Gaster M, Handberg A, Beck-Nielsen H, Schroder HD. Glucose transporter expression in human skeletal muscle fibers. Am J Physiol Endocrinol Metab. 2000;279:E529–38.
Guillemain G, Loizeau M, Pincon-Raymond M, Girard J, Leturque A. The large intracytoplasmic loop of the glucose transporter GLUT2 is involved in glucose signaling in hepatic cells. J Cell Sci. 2000;113:841–7.
Hackett SJ, Kimball RT, Reddy S, Bowie RCK, Braun EL, Braun MJ, et al. A phylogenomic study of birds reveals their evolutionary history. Science. 2008;320:1763–8.
Hazelwood RL. The avian endocrine pancreas. Am Zool. 1973;13:699–709.
Hedges SB, Kumar S. The timetree of life. New York: OUP Oxford; 2009.
Huang S, Czech MP. The GLUT4 glucose transporter. Cell Metab. 2007;5:237–52.
Irestedt M, Ohlson JI. The division of the major songbird radiation into Passerida and 'core Corvoidea' (Aves: Passeriformes)—the species tree vs. gene trees. Zool Scr. 2008;37:305–13.
Jetz W, Thomas GM, Joy JB, Hartmann K, Mooers AO. The global diversity of birds in space and time. Nature. 2012;491:444–8.
Jønsson KA, Bowie RCK, Moyle RG, Irestedt M, Norman JA, Fjeldså J. Phylogeny and biogeography of Oriolidae (Aves: Passeriformes). Ecography. 2010;33:232–41.
Katagiri H, Asano T, Ishihara H, Tsukuda K, Lin JL, Inukai K, et al. Replacement of intracellular C-terminal domain of GLUT1 glucose transporter with that of GLUT2 increases V_{max} and K_m of transport activity. J Biol Chem. 1992;267:22550–5.
Kono T, Nishida M, Nishiki Y, Seki Y, Sato K, Akiba Y. Characterisation of glucose transporter (GLUT) gene expression in broiler chickens. Br Poult Sci. 2005;46:510–5.
Leturque A, Brot-Laroche E, Gall ML. GLUT2 mutations, translocation, and receptor function in diet sugar managing. Am J Physiol-Endocrinol Metab. 2009;296:E985–92.
Long W, Cheeseman CI. Structure of, and functional insight into the GLUT family of membrane transporters. Cell Health Cytoskeleton. 2015;7:167–83.

MacDonald JR, Ziman R, Yuen RKC, Feuk L, Scherer SW. The database of genomic variants: a curated collection of structural variation in the human genome. Nucleic Acids Res. 2014;42:D986–92.
Maddison WP, Maddison DR. Mesquite: a modular system for evolutionary analysis. Version 3.61. 2019. https://www.mesquiteproject.org.
Moyle R, Taylor S, Oliveros C, Lim H, Haines CL, Abdul Rahman M, et al. Diversification of an endemic Southeast Asian genus: phylogenetic relationships of the spiderhunters (Nectariniidae: *Arachnothera*). Auk. 2011;128:777–88.
Mueckler M, Thorens B. The SlC2 (GLUT) family of membrane transporters. Mol Aspects Med. 2013;34:121–38.
Nicolson SW, Fleming PA. Nectar as food for birds: the physiological consequences of drinking dilute sugar solutions. Plant Syst Evol. 2003;238:139–53.
Osorlo-Fuentealba C, Contreras-Ferrat AE, Altamirano F, Espinosa A, Li Q, Niu W, et al. Electrical stimuli release ATP to increase GLUT4 translocation and glucose uptake via PI3K[gamma]-Akt-AS160 in skeletal muscle cells. Diabetes. 2013;62:1519–26.
Polakof S, Mommsen TP, Soengas JL. Glucosensing and glucose homeostasis: from fish to mammals. Comp Biochem Physiol B Biochem Mol Biol. 2011;160:123–49.
R Core Team. R: a language and environment for statistical computing. Vienna, Austria: R Foundation for Statistical Computing; 2020. URL https://www.R-project.org/.
Reding DM, Foster JT, James HF, Pratt HD, Fleischer RC. Convergent evolution of 'creepers' in the Hawaiian honeycreeper radiation. Biol Lett. 2009;5:221–4.
Rideau N, Derouet M, Grimsby J, Simon J. Glucokinase activation induces potent hypoglycemia without recruiting insulin and inhibits food intake in chicken. Gen Comp Endocrinol. 2010;169:276–83.
Roberts MW. Hummingbirds' nectar concentration preferences at low volume: the importance of time scale. Anim Behav. 1996;52:361–70.
Schuchmann K-L. Handbook of the birds of the world. Barcelona: Lynx Editions; 2015.
Seatter MJ, De la Rue SA, Porter LM, Gould GW. QLS motif in transmembrane helix VII of the glucose transporter family interacts with the C-1 position of D-glucose and is involved in substrate selection at the exofacial binding site. Biochemistry. 1998;37:1322–6.
Sedano RE, Burns KJ. Are the Northern Andes a species pump for Neotropical birds? Phylogenetics and biogeography of a clade of Neotropical tanagers (Aves: Thraupini). J Biogeogr. 2010;37:325–43.
Seki Y, Sato K, Kono T, Abe H, Akiba Y. Broiler chickens (Ross strain) lack insulin-responsive glucose transporter GLUT4 and have GLUT8 cDNA. Gen Comp Endocrinol. 2003;133:80–7.
Shen B, Han X, Zhang J, Rossiter SJ, Zhang S. Adaptive evolution in the glucose transporter 4 gene Slc2a4 in Old World fruit bats (family: Pteropodidae). PLoS ONE. 2012;7:e33197.
Shepherd PR, Kahn BB. Glucose transporters and insulin action—implications for insulin resistance and diabetes mellitus. N Engl J Med. 1999;341:248–57.
Sweazea KL, Braun EJ. Glucose transporter expression in English sparrows (*Passer domesticus*). Comp Biochem Physiol B Biochem Mol Biol. 2006;144:263–70.
Taniguchi CM, Emanuelli B, Kahn CR. Critical nodes in signalling pathways: insights into insulin action. Nat Rev Mol Cell Biol. 2006;7:85–96.
Thorens B. Glucose transporters in the regulation of intestinal, renal, and liver glucose fluxes. Am J Physiol-Gastrointest Liver Physiol. 1996;270:G541–53.
Thorens B. GLUT2 glucose sensing and glucose homeostasis. Diabetologia. 2015;58:221–32.
Thorens B, Mueckler M. Glucose transporters in the 21st Century. Am J Physiol-Endocrinol Metab. 2010;298:E141–5.
Uldry M, Thorens B. The SLC2 family of facilitated hexose and polyol transporters. Pflüg Arch. 2004;447:480–9.
Wals PA, Katz J. Glucokinase in bird liver a membrane bound enzyme. Biochem Biophys Res Commun. 1981;100:1543–8.
Warren BH, Bermingham E, Prys-Jones RP, Thébaud C. Immigration, species radiation and extinction in a highly diverse songbird lineage: white-eyes on Indian Ocean islands. Mol Ecol. 2006;15:3769–86.
Weir JT, Bermingham E, Schluter D. The Great American Biotic Interchange in birds. Proc Natl Acad Sci. 2009;106:21737–42.
Witteveen M, Brown M, Downs CT. Does sugar content matter? Blood plasma glucose levels in an occasional and specialist avian nectarivore. Comp Biochem Physiol Part A Mol Integr Physiol. 2014;167:40–4.
Workman RE, Myrka AM, Wong GW, Tseng E, Welch KC, Timp W. Single-molecule, full-length transcript sequencing provides insight into the extreme metabolism of the ruby-throated hummingbird *Archilochus colubris*. GigaScience. 2018;7:1–12.
Wright TF, Schirtzinger EE, Matsumoto T, Eberhard JR, Graves JR, Sanchez JJ. A multilocus molecular phylogeny of the parrots (Psittaciformes): support for a Gondwanan origin during the Cretaceous. Mol Biol Evol. 2008;25:2141–56.
Wu L, Fritz JD, Powers AC. Different functional domains of GLUT2 glucose transporter are required for glucose affinity and substrate specificity. Endocrinology. 1998;139:4205–12.
Zhang S, Yang L, Yang X, Yang J. Molecular phylogeny of the yuhinas (Sylviidae: *Yuhina*): A paraphyletic group of babblers including *Zosterops* and Philippine *Stachyris*. J Ornithol. 2007;148:417–26.
Zhang W, Sumners LH, Siegel PB, Cline MA, Gilbert ER. Quantity of glucose transporter and appetite-associated factor mRNA in various tissues after insulin injection in chickens selected for low or high body weight. Physiol Genomics. 2013;45:1084–94.
Zhao F-Q, Keating AF. Functional properties and genomics of glucose transporters. Curr Genomics. 2007;8:113–28.
Zuker M. Mfold web server for nucleic acid folding and hybridization prediction. Nucleic Acids Res. 2003;31:3406–15.