Plasma Membrane Recruitment Patterns of Glucose Transporters (GLUT) 1, 2, 3, and 5 in Response to Feeding in the Ruby-throated Hummingbird, *Archilochus colubris.*

by

Raafay Syed Ali

A thesis submitted in conformity with the requirements
for the degree of Master of Science

Department of Cell and Systems Biology
University of Toronto

Plasma Membrane Recruitment Patterns of Glucose Transporters (GLUT) 1, 2, 3, and 5 in Response to Feeding in the Ruby-throated Hummingbird, *Archilochus colubris*.

Raafay Syed Ali
Master of Science
Department of Cell and Systems Biology
University of Toronto
2018

Abstract

Hummingbirds, among the smallest vertebrate endotherms, have high resting and active metabolic rates and subsist almost exclusively on nectar sugar; they face extreme challenges to energy homeostasis and blood sugar regulation. Transmembrane sugar transport, and thus some control over energy flux, is mediated by facilitative glucose transporters, GLUTs. I determined relative protein abundance and subcellular localization via Western Blot for several GLUTs in flight muscle and liver from fed or fasted ruby-throated hummingbird (*Archilochus colubris*). I observed GLUT5 protein in flight muscle PM, consistent with its hypothesized uniquely high flux of fructose. GLUT1 protein was absent in hepatocytes, contradicting previously observations of transcript. Finally, glucose-specific GLUT1 abundance was increased in flight muscle PM of fed birds, while liver PM was increased in GLUT3 and glucose/fructose-transporting GLUT2. This suggests muscle glucose uptake capacity is dynamically regulated in response to feeding, while both glucose- and fructose-uptake capacity respond in the liver.

Acknowledgements

I would like to thank Dr. Kenneth C. Welch Jr. for his endless support and constant faith in me and my work. I would like to thank him for imbuing me with the same enthusiasm that continues to drive him to ask questions about the animals that inhabit our shared world. I would also like to thank my family, who make up the other side of the immense support I received to complete this project. Their never-ending love, patience, and homecooked meals gave me the freedom to pursue my interest in studying biological systems.

I would also like to thank my current and former lab members, without whom I could not have gotten this project off the ground. They kept me sane throughout the entire endeavour; sharing laughs, pranks, and even actual science. I would like to thank Alex Myrka for setting me up on a solid foundation to perform my experiments. And Dr. Morag Dick for her critical insights and guidance for this project. I would like to thank Lily Hou, Erich Eberts, Simon English, and Natalia Sandoval-Herrera for indulging in my crazy theories and providing the best working environment a graduate student could ask for.

I would like to thank my committee members, Dr. Blake Richards and Dr. Mauricio Terebiznik and also Dr. Aarthi Ashok for their expertise and evaluation of critical components of my project, and Dr. Stephen G. Reid for getting me started with the entire degree. I would also like to thank the collective research groups of Dr. Rene Harrison, Dr. Behbinn Treannor, and Dr. Rudy Boonstra for providing me with the tools I needed, sometimes in the nick of time.

I can’t possibly list everyone who contributed to this project, I honestly could not have done it myself. I am thankful to the administration keeping my accounts and funds in check, the department for facilitating this level of research, and the University for setting up such a welcoming and world-class research facility. And I also would like to thank you, the reader, for actually spending the time to go through what I’ve been up to for two years. Thank you.

Table of Contents

List of Tables

List of Figures

List of Appendices

List of Abbreviations

µL. *Microlitres*
AM. *Anno meridian*
ATP. *Adenosine triphosphate*
BSA. *Bovine serum albumin*
CA. *California*
ddH_2O. *double-distilled water*
DTT. *Dithiothreitol*
EDTA. *Ethylenediaminetetraacetic acid*
g. *Gravitational force*
GAPDH. *Glyceraldehyde-3-phosphate dehydrogenase*
GLUT. *Facilitative glucose transporter*
IR. *Insulin receptor*
K_m *Michaelis-Menten constant*
M. *Molar*
MCU. *Muscle carbohydrate uptake*
mM. *millimolar*
mRNA. *Messenger ribonucleic acid*
NaCl. *Sodium chloride*
NC. *Nitrocellulose*
NP-40. *Nonidet P-40*
O_2. *Oxygen*
˚C. *Degrees celcius*
OTC. *Oxygen transport cascade*
PBST. *Phosphate-buffered saline with Tween-20*
PCR. *Polymerase chain reaction*
PI3K. *phosphoinositide-3-kinase*
PM. *Plasma membrane*
POI. *Protein of interest*
PPM. *Post-plasma membrane*
RER. *Respiratory exchange ratio*
RIPA. *Radioimmunoprecipitation assay*
SDS. *Sodium dodecyl sulfate*
SDS-PAGE. *Sodium dodecyl sulfate polyacrylamide gel electrophoresis*
SGLT. *Sodium-linked glucose transporters*
SOC. *Sugar oxidation cascade*
TEMED. *Tetramethylethylenediamine*
USA. *United States of America*
UTSC. *University of Toronto Scarborough*
VLDL. *Very low-density lipoproteins*
$\dot{V}_{O_2}$. *Volume of oxygen uptake*
$\dot{V}_{O_2max}$. *Maximal oxygen uptake capacity*
$\dot{V}_{max}$ *Maximum rate of reaction*
VWR. *VWR International Co.*
W. *Watts*

Chapter 1

Introduction

1. Hummingbird Nectarivory.

1.1 Daily energy balance.

Hummingbirds subsist primarily on nectar from flowers (Powers & Nagy, 1988). The energy obtained from the cellular metabolism of these carbohydrates allows hummingbirds to perform one of the most energy-demanding behaviours known amongst vertebrates; hovering (Lasiewski, 1963). Hovering, in turn, allows hummingbirds access to sucrose-rich nectar that had only been available to bees or butterflies in the New World (del Rio, Baker, & Baker, 1992). As such, their physiology shows remarkable adaptations for the exploitation of such a niche, regulating monosaccharide transport from the gut to active cells in a manner that provides sufficient energy for their daily foraging behaviour while avoiding the perils that come from hyperglycaemia (Beuchat & Chong, 1998; Suarez & Welch, 2017).

Such extreme energetic behaviour requires a consistent supply of fuel. Over a full day of foraging, ingested nectar can provide approximately 1.6kJ (0.45W) of energy per hour in wild rufous hummingbirds, *Selasphorus rufus*, weighing ~2-5 grams (Gass & Sutherland, 1985; Suarez, 1992). In the wild, however, nectar availability is not always guaranteed. The replenishment rate of flowers and competition from other nectarivores can lead to periods of complete nectar unavailability (Gass & Sutherland, 1985). During these fasted states, blood glucose concentrations in the ruby-throated hummingbird, *Archilochus colubris*, falls to ~17mM, much higher than that of healthy postprandial human, but not remarkable compared to other birds' fasted blood glucose (Beuchat & Chong, 1998). With feeding, blood glucose rises to ~42mM; a glycaemic state much higher than in any other active vertebrate (Beuchat & Chong, 1998). How are hummingbirds capable of managing such dramatic glycaemic shifts while maximising both extraction and storage of energy? Their metabolic extremes allude to a physiology specialised for dramatic energetic balance, one in which rapid transitions occur in a daily manner (Powers & Nagy, 1988; Suarez et al., 1990). In this thesis, I will report on aspects of the early regulatory steps in the carbohydrate transport of the ruby-throated hummingbird, *Archilochus colubris*.

Extended periods of nectar unavailability require hummingbirds to shift to another source of fuel; stored fat. Hummingbirds produce fat stores primarily through *de novo* hepatic lipogenesis using ingested sugars (Hargrove, 2005; Suarez, Brown, & Hochachka, 1986). Each night, when their foraging period comes to an end, so does their steady supply of nectar. Once the final nectar meals of the day are metabolised, roosting hummingbirds must rely on endogenous fat stores to meet their overnight metabolic requirements. During their overnight fast, respiratory exchange ratios (RER, the ratio of carbon dioxide expiration rate to oxygen inspiration rate) are consistently ~0.71, indicating fatty acids are the primary metabolic fuel (Suarez et al., 1990; Welch, Bakken, del Rio, & Suarez, 2006). Migrating hummingbirds also rely on fat stores to power long-distance flight (Carpenter & Hixon, 1988; McWilliams, Guglielmo, Pierce, & Klaassen, 2004; Suarez et al., 1986). Fat stores can account for as much as 46% of a pre-migratory hummingbird's overall mass (Calder, 1994; Hargrove, 2005).

Despite ample fat-stores, hummingbird physiology appears to prefer carbohydrate metabolism over that of fatty-acids. After rousing from overnight sleep, and following a few of bouts of hover-feeding for nectar, hummingbird RER values rise sharply to ~1.0 (Suarez, 1992; Suarez et al., 1990; Welch et al., 2006). This indicates that carbohydrates are the primary metabolic fuel during hovering. However, even if perched and sedentary, hummingbirds that are provided a sucrose solution following a fast have an RER ~1.0 (Suarez, 1992). The postprandial increase in the abundance of circulating sugars may be enough to initiate a metabolic fuel source switch in hummingbirds.

1.2 Carbohydrate management.

Hummingbird blood sugar regulation breaks from our understanding of carbohydrate management in mammals. In mammals, the likelihood that a 6-carbon monosaccharide, i.e. a hexose molecule, is directed into pathways for immediate metabolism is determined by exercise status. As an exercising mammal approaches its maximal oxygen uptake capacity ($\dot{V}_{O_2max}$), it relies less on fat oxidation (Roberts, Weber, Hoppeler, Weibel, & Taylor, 1996) and increasingly on carbohydrate oxidation (Weber, Roberts, Vock, Weibel, & Taylor, 1996).

It is difficult to compare an exercising bird to an exercising mammal. While an exercising mammal can modulate the relative contribution of fats and carbohydrates by adjusting their $\dot{V}_{O_2}$

(for example, a running human can slow down their running pace in an effort to promote greater utilisation of fatty acid stores), forward flying birds are unable to make the same adjustment, requiring a minimum sustained oxygen consumption for flight to occur successfully; a $\dot{V}_{O_2}$ that's already twice as high as similarly sized running mammal (McWilliams et al., 2004). Furthermore, unweighted forward flying birds do not fly while operating near $\dot{V}_{O_2 max}$, only reaching 60-85% of their maximal aerobic capacity (McWilliams et al., 2004).

Overall, mammals operating at >40%$\dot{V}_{O_2}$ derive 60-70% of their total energy from intra-muscular glycogen and only 13-23% from circulating carbohydrates (Weber, 2011; Weber et al., 1996). This is vastly different in hummingbirds who derive 90-95% of energy required for hovering flight from recently ingested carbohydrates (Welch, Altshuler, & Suarez, 2007) and will not support sustained glycogenolysis at any exercise level (Suarez et al., 1986).

The mammalian model, in this context, fails to describe the fuel-use of flying hummingbirds. Hummingbird carbohydrate management is unique in that the active cells' primary metabolic fuel source is not dictated by exercise-status, but dietary status (Welch, Myrka, Ali, & Dick, 2018). Hummingbird cells show preference for the metabolism of the recently ingested carbohydrates as they become available, even if they are not actively flying (Welch et al., 2006). Remarkably, the switch to carbohydrate metabolism can occur in as little as 20 minutes of the initial sucrose ingestion (Suarez, 1992; Suarez et al., 1990; Welch et al., 2006).

An energetics perspective highlights the advantage hummingbirds have with their exclusive reliance on recently ingested carbohydrates compared to the mixed fuel-use observed in most mammals. In vertebrates, carbohydrate metabolism allows for more efficient energy-production of the oxygen transported to active cells. The number of adenosine triphosphate (ATP) molecules produced per molecule of oxygen, known as the P/O ratio, differs depending on the substrate being metabolised. In hummingbirds, the P/O ratio is 15% higher if the primary metabolic substrate is derived from recently ingested carbohydrates, rather than previously stored lipids (Welch et al., 2007). Furthermore, they are then able to resort to these fat-stores in times of energy deficiency, leaving circulating carbohydrates to fulfill immediate fuel demands. However, this does not speak to the abundance of fuel available for metabolism, only that energy-transport is most efficient if ingested carbohydrate molecules are immediately oxidised. How, then, do hummingbird maximise oxygen and carbohydrate delivery to active cells to

facilitate some of the highest metabolic rate per unit mass observed amongst vertebrates (Suarez, 1992; Welch et al., 2018)?

1.3 The Sugar Oxidation Cascade.

Once ingested, carbohydrate transport within the hummingbird occurs largely in parallel to oxygen transport. The barriers to oxygen transport from ingestion to combustion exist as a series of resistances which oxygen molecules must overcome. These include 1) transport into the cardiovascular system, 2) transport to extracellular environment of active cells, and 3) transport across biological membranes into active cells and subsequent oxidation (Di Prampero, 1985). Much work has been done to understand how hummingbirds achieve such high rates of flux through the oxygen transport cascade (OTC; Di Prampero, 1985) and these involve adaptations at each of these steps that enhance capacity for, and represent regulatable sites of control over oxygen flux (Suarez, 1998). Thus, some of the adaptations that enhance flux through the OTC will have similar effects on carbohydrate transport. This parallel transport is known collectively as the Sugar Oxidation Cascade (SOC) (Suarez & Welch, 2011).

Together, the OTC and SOC provide the substrates necessary for carbohydrate oxidation at rates sufficient to support the exceptional metabolic rates associated with foraging and hovering flight. The differences in OTC and SOC lie primarily in the initial absorption and final transmembrane transport mechanics of the hexoses themselves and will be discussed in detail below.
Key steps of the SOC differ from those of the OTC in ways that have functional consequences (Suarez & Welch, 2011). While oxygen molecules may move from outside to inside active cells by simple diffusion, hexoses cannot do so. Glucose transporters (GLUTs) allow passage of hydrophilic hexoses through hydrophobic domains of eukaryotic plasma membranes (PM) (Alves, Almeida e Silva, & Giulietti, 2007; Thorens & Mueckler, 2010; Uldry & Thorens, 2004) and are thus an essential part of this distributed control over total carbohydrate flux through a vertebrate organism. GLUT presence or absence in the plasma membrane (PM) of active cells has a regulatory function; the presence of specific isoforms, their abundance, and their transport kinetics effectively determine transport capacity of hexoses into and out of an active cell (Wasserman, 2009). It is critical to regulate the transport of recently ingested hexoses as nectarivores must reduce the possibility of glucotoxicity from excessive circulating glucose, such

as (Kaiser, Leibowitz, & Nesher, 2003; Solomon et al., 2012) without compromising fuel availability.

In mammals, insulin is the primary signal for the mammalian increase in plasma membrane (PM) GLUT abundance, particularly that of GLUT4 (Guma, Zierath, Wallberg-Henriksson, & Klip, 1995). Hummingbirds, much like all birds, have muscles that are insulin-resistant and lack GLUT4 (Dupont, 2009; Welch, Allalou, Sehgal, Cheng, & Ashok, 2013; Workman et al., 2018). However, known GLUT-isoforms are sensitive to other signals as well, including changing blood carbohydrate concentrations (Mathoo, Shi, Klip, & Vranic, 1999). The consensus mammalian and avian tissue-specific distribution of GLUT-isoforms is summarised in Appendix 1 and Appendix 2, respectively, and will be discussed below in their tissue-specific context. Considering the differing combinations and regulation of GLUT-isoforms present on avian cells (Byers, Howard, & Wang, 2017), and considering the more extreme nature of shifting hummingbird glycaemia, it follows that GLUT regulation, while critical, may be different in the hummingbird system compared to mammals (Wagstaff & White, 1995).

2. Carbohydrate transport in the hummingbird

2.1 Transport across the gut.

After a short time (10-30 minutes) spent in a small crop (~500µL), consumed nectar makes its way to the hummingbird gut (Hainsworth & Wolf, 1972). Sucrose is the primary carbohydrate in the nectar of hummingbird-pollinated plants (del Rio et al., 1992) and is largely inaccessible by gut enterocytes. It is cleaved into its constituent hexose monosaccharides, glucose and fructose, by the rapid sucrase enzyme found in enterocyte brush borders (del Rio et al., 1992). The interaction of sucrase enzyme with sucrose is facilitated by a higher lumenal surface area of the hummingbird gut compared to mammals, in part due to greater villous amplification (Price, Brun, Caviedes-Vidal, & Karasov, 2015).

It is a combination of facilitated glucose transporters (GLUTs) and sodium-linked glucose transporters (SGLTs) that constitute the active and regulatable transmembrane hexose transport (Karasov, 2017; Price et al., 2015). While the vast majority of research surrounds that of mammalian GLUTs (summarised in Appendix 1), their high degree of functional conservation observed with those of avian systems (summarised in Appendix 2) provides a useful starting

point for describing the behaviour of avian GLUTs. Sodium glucose cotransporters (SGLTs) also allow passage of carbohydrate across PMs. However, unlike GLUTs, these proteins co-transport two sodium ions for every hexose molecule (Scheepers, Joost, & Schurmann, 2004; Sweazea & Braun, 2006; Wood & Trayhurn, 2003). Enterocyte GLUT and SGLT isoforms are located at subcellular sites that allow them to easily access and rapidly transport their preferred substrate across the PM and into the enterocyte itself or further on into the cardiovascular system.

GLUT2 and SGLT1 are the main glucose transporters in the intestine (Scheepers et al., 2004; Wood & Trayhurn, 2003). GLUT2 has a relatively high K_m of ~17mM for glucose (Zhao & Keating, 2007). Yet, despite its low affinity, GLUT2 has a very high capacity for glucose transport (Thorens, 1996). SGLT1 is located on the apical side of enterocytes, and while it has a high affinity for glucose, it has a low capacity for its transport (Braun & Sweazea, 2008; Scheepers et al., 2004; Wood & Trayhurn, 2003). SGLT1 might play a more important role in postprandial glucose-sensing than transport. Glucose stimulates SGLT1 to recruit GLUT2 to the apical enterocyte membrane, resulting in a 3-fold increase in transmembrane carbohydrate transport than with SGLT1 transport on its own (Kellett, Brot-Laroche, Mace, & Leturque, 2008, p. 2).

GLUT2 can also transport fructose (K_m ~76mM) with low affinity. GLUT2 along with GLUT5 (K_m fructose ~10-12 mM) establish the facilitated fructose transport across enterocyte membranes (Byers et al., 2017; Uldry & Thorens, 2004). While GLUT5 has affinity for fructose transport, it is incapable of transporting glucose (Douard & Ferraris, 2008; Thorens, 1996). In rat jejunum, GLUT5 is found on the apical side of enterocytes and accounts for ~72% of fructose transport (Helliwell, Richardson, Affleck, & Kellett, 2000). However, following a meal, GLUT5 abundance was found to change minimally in the apical PM. Instead, GLUT2, although largely residing in the basolateral membrane, is recruited to the apical membrane and efficiently contributes to the remainder of fructose transport (Kellett et al., 2008; Thorens, 1996; Thorens & Mueckler, 2010). It must be noted, however, the apical PM recruitment of GLUT2 has not yet been clearly demonstrated in avian systems (Karasov, 2017).

Following cleavage, paracellular hexose transport between enterocyte tight junctions is thought to contribute to the majority of hexose transport across the hummingbird gut into extracellular space basal to the brush border before entering the bloodstream (McWhorter, 2006; Price et al.,

2015). While there may be some capacity to modulate the permeability of these tight junctions, they largely operate in a passive manner (Karasov, 2017). The increased villous surface area provides hummingbirds with the highest capacity for both active and passive hexose uptake observed amongst vertebrates (Karasov, 2017; McWhorter, 2006). These transporters, in combination with the gut enzymes and passive transport systems ensure a rapid break down of disaccharide and movement of constituent monosaccharide into the bloodstream. However, this is just the first step in the journey of sugars from ingestion to where they are catabolised; active cells such as the flight muscle. Hummingbirds must carefully partition the obtained sugars for both immediate use and long-term storage, and understanding this regulation requires observation of GLUT isoforms expressed in the PM of active cells themselves. The next step of SOC involves transport of these ingested sugars from the gut to the site of active cells.

2.2 Transport through the cardiovascular system

2.2.1 Circulating carbohydrates.

Hummingbird hearts beat at rates as high as 400-1200 per minute during flight (Didio, 1967; Lasiewski, 1964). Combined with a high stroke volume, their cardiac output is five times their body weight per minute and greatly enhances the per-minute delivery of blood (Lasiewski, 1963, 1964). Capillary density is also greatly enhanced, with a larger length per-fibre-volume that allows for greater capillary-to-fibre ratio than those seen in mammalian hindlimbs (Mathieu-Costello, Suarez, & Hochachka, 1992). Certain traits, such as a relatively higher hematocrit (56.3%) (Johansen, K, 1987), only enhance oxygen transport, however hexoses are able to dissolve into the blood directly (Alves et al., 2007). Oxygen itself is ingested at a rate of ~1.67μL per breath (Lasiewski, 1964; Suarez et al., 1986) However, as noted above, adaptations to oxygen transport simultaneously benefit carbohydrate transport (Suarez & Welch, 2011), and this is especially true with regards to transport through the circulatory system.

Exogenous circulating carbohydrates present with the additional problem of transiency; their levels are subject to dietary status. In most vertebrate cardiovascular systems, glycaemia exists between maximum and minimum concentrations of circulating hexoses. These concentrations represent the ‘fed’ and ‘fasted’ states, respectively, of the organism in question. The fasted state is characterised by a blood glucose concentration that is comparatively decreased from the post-

prandial, or fed, state as well as an increase in blood ketone levels (Belo, Romsos, & Leveille, 1976; Brady, Romsos, Brady, Bergen, & Leveille, 1978).

2.2.2 Reduced response to insulin in the muscle, but not the liver.

In mammalian systems, insulin, released from pancreatic β-cells, and glucagon, released from pancreatic α-cells, work to respectively decrease or increase blood glucose. These hormones travel through the cardiovascular system and communicate with organs such as the muscles, liver, and pancreas which work to import circulating carbohydrates for metabolic, glycogenic, lipogenic purposes (Klip & Vranic, 2006). This regulation is very strict, ensuring an adult male humans weighing approximately 70kg has no more or less than 4g of glucose in their circulatory system (Wasserman, 2009).

While little work has been done on the insulin-glucagon axis in hummingbirds, birds such as the granivorous chicken, *Gallus gallus*, have similar circulating insulin levels compared to mammals (Dupont, 2009). However, it is only chicken liver that is sensitive to insulin; the muscles seem resistant (Dupont, 2009). Chickens lack the insulin-recruitable skeletal muscle glucose transporter, GLUT4 at the mRNA and protein level (Braun & Sweazea, 2008; Chen, 1945; Dupont, 2009). Chickens are also missing this isoform at the genomic level (Seki, 2003). Crucially, GLUT4 is missing in the hummingbirds; showing no detectable mRNA or protein expression in ruby-throated hummingbirds (Welch et al., 2013; Workman et al., 2018) and no genomic presence in the recently mapped Anna's hummingbird, *Calypte anna*, genome (Zhang et al., 2014) .

Insulin signalling in any organ requires the presence of all appropriate cell-surface insulin receptors (IRs) and secondary signalling cascades. The typical mammalian response to insulin binding to the IR results in the transient activation of phosphoinositide 3-kinase (PI3K) which leads to glucose transport, glycogen synthesis, and lipid synthesis through activation of the Akt (protein kinase B) pathway. This pathway also acts as an inhibitory feedback mechanism in mammals, with increasing PI3K levels inhibiting insulin receptor substrates (Dupont, 2009). As noted previously, the chicken liver shows insulin-sensitivity and expresses cell-surface IRs along with functioning secondary messengers (Dupont, 2009). IRs are also present in chicken muscle cells (Dupont, 2004), however, muscle activity is chronically upregulated 30-fold, resulting in the over-stimulation of the inhibitory pathway leaving chicken muscle largely insulin-insensitive

(Dupont, 2009). Insulin may also act on avian kidneys, causing hypoglycaemia through an increased glomerular filtration however this has only been demonstrated in mourning doves, *Zenaida macroura*, (Sweazea, Braun, & Sparr, 2017). While the presence of IRs has not yet been described in hummingbird flight muscle, avian systems as a whole are considered largely insulin-resistant, requiring much greater than physiologically-relevant doses to demonstrate convulsivity (Chen, 1945). Conversely, avian systems much more sensitive to glucagon than mammals (Braun & Sweazea, 2008; Hazelwood, 1973). Produced in pancreatic α-cells, increasing glucagon levels result in increases in blood glucose, triglyceride, glycerol and free-fatty acid levels (Braun & Sweazea, 2008). Hummingbird glucagon warrants further study as well, especially as avian glucagon has been shown to play a key role in endogenous gluconeogenesis during exercise (Wasserman et al., 1989).

2.2.3 Blood hyperglycaemia.

As a result of the reduced role of insulin and increased role of glucagon, nearly all birds are hyperglycaemic; blood glucose levels are approximately twice as high relative to a mammal of similar body size (Brady et al., 1978; Braun & Sweazea, 2008). This is especially true of hummingbirds where fasted blood glucose levels are already higher than the highest possible in healthy mammals, at ~17mM (Beuchat & Chong, 1998). Their postprandial blood glucose concentration are higher still at ~42mM (Beuchat & Chong, 1998). Hummingbird blood fructose levels in either dietary state are currently unknown (Welch et al., 2018). Normal mammalian blood fructose levels are generally quite low, at ~0.1-1mM, and the hexose is rapidly incorporated into glycogenic and gluconeogenic pathways (Feinman & Fine, 2013).

Unlike mammals, hummingbird metabolic physiology demonstrates ambivalence towards glucose or fructose, utilising either for hovering (Chen & Welch, 2014). This suggests a regulatory capacity for fructose that is as capable as that of glucose, shifting away from the typical mammalian muscle model that prefers glucose (Feinman & Fine, 2013). Because the most dynamically-regulated transporter, GLUT4, is missing from the avian genome, my focus falls on the other GLUTs (1, 2, 3, and 5) to understand the transport capacity for glucose and fructose among energetically active tissues and how this capacity changes in relation to feeding. Below, I describe the patterns of GLUT protein expression and PM localization in the ruby-throated hummingbird liver and flight muscle.

2.3 Transport into active cells

Crucial to understanding how hummingbirds manage energy balance is elucidating capacities for sugar transport among tissues. Hummingbird cells have the capacity to rapidly phosphorylate recently ingested carbohydrates once they have crossed the PM and are within the cytosol of the active cell. Their muscle hexokinase enzyme $\dot{V}_{max}$ for glucose is 21.09 +/- 1.81 μmols min^{-1} g wet $tissue^{-1}$ and 12.94 μmols min^{-1} g wet $tissue^{-1}$ for fructose (Myrka & Welch, 2018). This phosphorylation capacity ensures that carbohydrate import into the cell is consistently favoured and sufficient for high metabolic demand (Suarez & Welch, 2017). Indeed, glucose phosphorylation rates meet calculated minimum requirements to sustain hovering flight (Suarez et al., 1986). However, work remains to be done addressing the discrepancy between their relatively lower fructose phosphorylation rate and their ability to hover solely on recently ingested fructose (Chen & Welch, 2014; Myrka & Welch, 2018).

The mammalian carbohydrate transport system is regulated by three major players; the pancreas, liver, and muscle (Klip & Vranic, 2006; Wasserman, 2009). In the avian carbohydrate transport system, the pancreas has a reduced hypoglycaemic role, while a greater proportion of regulation can be attributable to the active muscles and liver. Avian pancreatic insulin and glucagon release differs in the manner described above. Accordingly, the organ exhibits many more α-cells than β-cells (Braun & Sweazea, 2008; Pollock, 2002). The pancreatic release of insulin in response to feeding or fasting, i.e. glucose, is also reduced, with β-cells showing greater sensitivity to compounds such as cholecystokinin, glucagon, and some amino acids (Pollock, 2002). As such, and because of the small size of the tissue made collection logistically unfeasible, I assumed a lesser contribution of the hummingbird pancreas for the purposes of this study.

2.3.1 The Hummingbird Liver

The avian liver is the site of *de novo* lipogenesis (McWilliams et al., 2004; Ramenofsky, Savard, & Greenwood, 1999), a role that is largely reduced in avian adipocytes (Griffin, Guo, Windsor, & Butterwith, 1992). The postprandial avian liver exports fats for storage in adipocytes and exports triglyceride-rich very low density lipoproteins (VLDLs) for use by active muscles (Ramenofsky et al., 1999). Stored fat is essential for hummingbird long-distance migratory behaviour (Suarez et al., 1986). This is most clearly observed during hummingbird pre-migratory fattening where individuals may add ~40% in mass over just four days; an increase almost

entirely attributable to enlarged adipose stores (Carpenter, Hixon, Beuchat, Russell, & Paton, 1993; Hou, Verdirame, & Welch, 2015). To maximise fat stores, the postprandial liver becomes a site for net carbohydrate import leading to hepatic lipogenesis (Dupont, 2009; McWilliams et al., 2004), a process that may be stimulated by insulin (Pencek et al., 2003).

The hummingbird liver has been shown to express mRNA of GLUT isoforms 1, 2, 3 and 5 mRNA transcript (Myrka & Welch, 2018; Welch et al., 2013; Workman et al., 2018) though little is known about protein-level expression. As such, inferences must be made from the numerous mammalian and chicken studies on the topic.

GLUT1 in the chicken liver only shows mRNA expression; no actual protein expression occurs (Carver, Shibley, Jr, Pennington, & Pennington, 2001). The primary hepatic GLUT isoform in chickens is GLUT2 (Byers et al., 2018; Thorens, 1996). As previously noted, GLUT2 has a very high capacity for glucose and fructose transport and is involved in the rapid equilibration of the hexoses between the cytosolic and extracellular environments (Burcelin & Thorens, 2000; Thorens, 1996). Protein for the fructose-transporting GLUT5 and the high glucose affinity GLUT3 is also found in mammalian hepatocytes (Karim, 2012). Combined with the rapid phosphorylation capacity of the liver for both glucose and fructose (Bode, Bode, Ohta, & Martini, 1980), it is evident that the vertebrate liver is set up for high capacity processing of both hexoses present in the hummingbird diet (Powers & Nagy, 1988).

2.3.2 The Hummingbird Flight Muscles.

Muscle carbohydrate uptake (MCU) is dependent on the availability of carbohydrates in the muscular interstitium as well as the carbohydrate-phosphorylation capacity of the active myocyte (Wasserman, 2009). The flight muscles, the pectorals and supracoracoideus, represent a larger proportion of the total mass of hummingbirds (25-30%) compared with other avian species (average ~17%) (Mathieu-Costello et al., 1992). Furthermore, their locomotor muscle mitochondria consume 90% of available metabolic fuel during intense exercise. (Suarez, Lighton, Brown, & Mathieu-Costello, 1991). They comprise ~35% of the volume of hummingbird flight muscle fibres (Mathieu-Costello et al., 1992) and with highly-folded cristae that allow for 7-10mL of O_2 to be consumed per cm^3 of mitochondria per minute – twice as high as mammalian systems (Suarez et al., 1991).

Just as hummingbird flight muscles are relatively larger, they are also more vascularised and present with a high capillary-to-muscle-fibre ratio compared to mammals (Mathieu-Costello et al., 1992). This leads to a reduced distance that carbohydrates have to travel between capillaries and muscle interstitium, allowing the carbohydrate pool to be readily accessible (Suarez, 1998). Recent work on modelling muscle interstitium carbohydrate concentrations *in silico* show that this increased capillary network contributes to the pool of carbohydrates immediately available to active myocytes following a meal (Sové, Goldman, & Fraser, 2017); local glucose concentrations around capillaries were significantly higher than those parts of the interstitium that were further away. Furthermore, the *in silico* tests using computation software corroborate the importance of capillary density to MCU, with the densest simulations showing 35% higher glucose uptake rates compared to the least dense (Sové et al., 2017). Together with the previously described increased mitochondrial upregulations, these muscle adaptations result in a high capacity for both oxygen and carbohydrate import (Suarez, 1998).

It is important to note that hummingbird flight muscles have very low glycogen stores; ~30µmols in a 4g hummingbird (Didio, 1967; Suarez et al., 1990). it has been calculated that stored glycogen cannot power hovering flight for more than 5 minutes, necessitating a switch to newly available fuel, such as the high levels of circulating carbohydrates (Suarez et al., 1990). As such, while the above gastro-respiro-cardio-vascular adaptations greatly enhance the presence of recently ingested carbohydrates at sites of active cells, active muscles themselves play an enormous role in hummingbird utilisation and regulation.

3. Regulation of carbohydrate flux.

The sugar oxidation cascade provides multiple and disparate control systems at each of the barriers to hexose movement through biological systems (Suarez & Welch, 2011). Hummingbirds, much like other vertebrate nectarivores, possess adaptations at each of these barriers, enabling efficient shuttling of carbohydrates to their point of incorporation into metabolic pathways (Suarez & Welch, 2017). GLUTs are part of this distributed network of glycaemic control. Their expression and abundance in the PM of active cells regulates transmembrane hexose transport (Wasserman, 2009). It is critical to regulate the import of hexoses as vertebrates must reduce the possibility glucotoxicity without compromising fuel availability (Solomon et al., 2012).

GLUTs function to facilitate carbohydrate import into an active cell. A hypothetical cell that is completely permeable to hexoses may lead to uncontrolled and continuous metabolism as imported hexoses are phosphorylated. The impermeability of the PM to hexoses functions to reduce this maximal hexose import and continuous incorporation through the glycolytic pathway. GLUTs act as rate-enhancing proteins; to be upregulated in PM abundance when hexose import is required (Wasserman, 2009). In hummingbird muscle cells, imported glucose and fructose are phosphorylated at rates much higher than that of mammals, although a clear preference for glucose is exhibited by muscle hexokinase (Myrka & Welch, 2018). Indeed, phosphorylation of glucose is irreversible in muscle; glucose-6-phosphate acts to reduce hexokinase activity, allowing for a disparate control system for glucose flux through glycolysis (Wasserman, Kang, Ayala, Fueger, & Lee-Young, 2011). Furthermore, this 'locking-in' of hexoses creates a hexose gradient which further favours glucose, and possibly fructose, import.

Hummingbird glycaemic regulation breaks with the mammalian model in two important ways. Firstly, and as noted previously, hummingbird muscles are insulin-resistant and lack GLUT4 (Dupont, 2009; Welch et al., 2013; Workman et al., 2018). However, GLUT-isoforms are sensitive to other signals as well, including changes in blood carbohydrate concentrations (Mathoo et al., 1999). Considering the differing combinations and regulation of isoforms present on avian cells (Byers et al., 2017), and considering the more extreme nature of shifting hummingbird glycaemia, it follows that GLUT utilisation, while critical, may be different in the hummingbird system (Wagstaff & White, 1995).

The second is the exclusive use of recently ingested carbohydrates as the primary metabolic fuel source in hummingbirds in both their muscles and liver. While it may seem unintuitive that a potent signal for glucose uptake, insulin, has such a reduced role in an extremely sugar-hungry tissue, the muscles, it suggests that hummingbirds may regulate their muscle GLUT isoforms differently from that of the insulin-sensitive liver. The stimulus for GLUT regulation in the liver may be consistent with the mammalian model and depend on insulin, but the insulin-insensitivity of the muscles indicates the stimulus may be the presence of carbohydrates themselves. What GLUT-isoforms are present in at the protein level in the PM of hummingbird cells? Furthermore, which of these isoforms response to being fed a sucrose meal?

4. Hypotheses and Predictions

To better understand the family of proteins responsible for transmembrane sugar transport, I explored the PM and cytosolic abundance of GLUT1, 2, 3, and 5 in the flight muscle and liver of ruby-throated hummingbirds. I hypothesised that I would observe protein of GLUT isoforms for which mRNA or protein was previously observed. As mRNA for GLUT1 and 5 had been detected in hummingbird flight muscle (Myrka & Welch, 2018; Welch et al., 2013), as well as protein for GLUT2 and GLUT3 in mammalian muscle (see Appendix 1), I predicted that I would observe the protein presence of these GLUT isoforms in ruby-throated hummingbird flight muscle. As well as this, mRNA transcript for GLUT1, 2, 3, and 5 were observed in the recently-sequenced ruby-throated hummingbird liver transcriptome (Workman et al., 2018); I predicted I would also observe protein for GLUT1, 2, 3 and 5 in the ruby-throated hummingbird liver.

In order to understand how nectarivores manage large and dynamic changes in circulating sugar concentration, I compared the GLUT-protein abundance in hummingbirds that had been fed a sucrose meal in comparison with ones that were fasted for 1 hour. While fructose-only meals are sufficient to power hummingbird hovering flight (Chen & Welch, 2014), the relatively lower phosphorylation capacity of hummingbird muscle for fructose (Myrka & Welch, 2018) may reflect a reduced role of fructose in the muscles when glucose is also present. I predicted that I would see a relatively higher abundance of glucose-specific GLUT isoforms in the flight muscle PM of fed hummingbirds. With the understanding that GLUT1 and GLUT3 show the highest affinity for glucose as their substrate, I predicted that I would observe the greatest increase in flight muscle PM-GLUT1 and PM-GLUT3 abundance which may reflect an increase in the import capacity of glucose, rather than fructose following a sucrose meal.

GLUTs also play a strong role in the post-prandial liver, allowing rapid influx of both glucose and fructose for incorporation into lipogenic, gluconeogenic, and glycogenic pathways. With the postprandial liver showing high glucose and fructose import rates (Bode et al., 1980), along with the reduced role of avian hepatic GLUT1 , I predicted that GLUT3 and GLUT5 will show the greatest increase in PM abundance of hummingbird hepatocytes following a meal. This prediction also rests on the understanding that GLUT3 specifically increases glucose transport and GLUT5 fructose transport, making up the entirety of hepatic carbohydrate transport following a sucrose meal.

Chapter 2
Procedure and Results

1. Materials and methods

1.1 Animal use and ethics statement.

This study was approved and performed adhering to the requirements of the University of Toronto Laboratory Animal Care Committee as well as the Canadian Council on Animal Care.

Adult male ruby-throated hummingbirds (*Archilochus colubris*) were captured at the University of Toronto Scarborough (UTSC) using modified box traps. The hummingbirds were housed individually in Eurocages (Corners Ltd, Kalamazoo, MI, USA) in the UTSC vivarium facilities. They were provided with perches and fed on a maintenance diet of NEKTON-Nectar-Plus (Keltern, Germany).

To perform the fed and fasted treatments, hummingbirds were removed from their housing cage in the morning (10AM) and placed in a 1-litre jar with limited room for movement. The fed group was provided with *ad-libitum* 1M sucrose solution for 1 hour. The fasted group was deprived of any food for 1 hour. This was previously demonstrated by (Chen & Welch, 2014) to be more than sufficient time to ensure the primary metabolic fuel of fed hummingbirds was carbohydrates, while fasted must resort to fatty-acid catabolism. Following the 1-hour period, hummingbirds were anaesthetised with isofluorane inhalation. Their state of consciousness was determined using the eye-blink test and were euthanised with decapitation. Hummingbird pectoralis and supracoracoideus muscle was obtained as the flight muscle sample and placed in isopentane cooled with liquid nitrogen. The liver was obtained approximately 1-2 minutes following decapitation and was frozen in a similar manner. Tissues were stored at -80^{O}C.

1.2 Antibody design and production.

I obtained polyclonal antibodies from Pacific Immunology (Ramona, CA, USA). mRNA sequences for hummingbird GLUT isoforms 1, 2, 3, and 5 were obtained from ruby-throated hummingbird liver transcriptome (Workman et al., 2018). The antibody manufacturer assessed the sequences for regions of greatest dissimilarity between isoforms and used them to develop a synthetic peptide to act as an immunogen. ~20-amino acid long oligomers that were injected into

rabbits, along with the appropriate adjuvants, and antibody was developed for ~6 months. Their serum was harvested twice before a final lethal exsanguination. The serum was affinity-purified, its concentration assessed using ELISA, and the antibody was frozen and shipped to me.

1.3 Sample preparation for subsequent protein analysis – initial attempts

My initial attempts at SDS-PAGE and subsequent Western blotting involved homogenisation methodology developed previously by Velten & Welch (2014). Unfortunately, samples prepared via this protocol led to problems of high-molecular weight aggregations with downstream Western blotting techniques (Appendix 4). This was most likely a result of the use of a tissue tearer as well as a lack of initial centrifugation. As such, although it is briefly mentioned here, the protocol I assumed was a modified version of Yamamoto et al. (2016) which I detail below.

All reagents were prepared and cooled before homogenisation. All homogenisation and centrifugation steps were carried out on ice or at 4^{o}C unless otherwise noted.

50mg of frozen tissue was weighed to which 500µL of cold urea lysis buffer (150mM NaCl, 0.1% Triton X-100, 5mM sodium deoxycholate, 0.001% SDS, 5mM Tris HCl (pH 8), 5mM Ethylenediaminetetraacetic acid (EDTA), 8M Urea) was added. On ice, tissue was minced with sharp dissection scissors. Tissue was homogenized using 5 × 10-second bursts of VWR Tissue Tearer followed by 3 × 2-second bursts of VWR sonicator to liberate membrane-residing proteins. The homogenate was centrifuged (Beckman Coulter Microfuge22R Centrifuge, Brea, CA) at 13000*g* for 15 minutes at 4^{O}C. The supernatant was collected and stored for subsequent Western blot analysis. However, I discontinued the use of this method as it caused nonspecific protein aggregates that will be discussed in detail in the discussion.

1.4 New method of sample preparation.

To overcome the problem of unwanted protein aggregates, I modified a protocol established by (Yamamoto et al., 2016). My modification consisted of replacing NP-40 (nonidet P-40) prescribed by the protocol with Triton X-100. This was done as NP-40 is no longer available in North America, and Triton X-100 is a close alternative`, allowing for a similar critical-micellar-count (Li, Luo, & Liu, 2004); an essential component regulating formation of detergent-protein complexes. All other steps proceeded as dictated by the protocol.

Buffer A (0.5M DTT, ddH_2O) was synthesised from which two aliquots were prepared containing 0.1% Triton X-100 (Buffer AO1) and 1% Triton X-100 (Buffer A1). The detergent concentration in these solutions is critical to their function. 2X RIPA (20mM Tris-HCl, pH 8.0, 300mM NaCl, 2% v/v Triton X-100, 1% (w/v) sodium deoxycholate, 0.2% (w/v) sodium dodecyl sulfate (SDS), 1mM DTT) was also prepared. All reagents were and cooled to 4^{o}C before homogenisation and included Sigma P8340 protease inhibitor cocktail to minimise the digestive effect of lysosomal enzymes released during the procedure. All homogenisation and centrifugation steps were carried out on ice or at 4^{o}C unless otherwise noted.

20mg of flight muscle or liver was cut from frozen tissue samples of fed or fasted hummingbirds. Tissue was cut on an aluminum block cooled with liquid nitrogen and placed in an ice-bath. The tissue was minced for 5 seconds with sharp dissection scissors. 180µL of cold buffer A01 was dispensed onto the sample and mincing was resumed for 15 seconds. Still on ice, tissue was homogenised using a VWR handheld pestle homogenizer (BELAF650000000) for 10-15 seconds. The homogenate was passed through a 21G needle three times to liberate nuclear and intracellular proteins. A 30µL aliquot was transferred to a fresh 1.5mL tube and an equal volume of 2x RIPA buffer was added. This was left on ice for 60 minutes with frequent, but gentle, agitation. The RIPA-sample was then centrifuged at 12,000*g* for 20 minutes at 4^{o}C. The supernatant was collected and stored at -80^{o}C as the whole-cell lysate, or ‘cell-fraction’.

1.4.1 Plasma membrane fractionation

The remainder of the homogenate from the above step was centrifuged at 200*g* for 1 minute at 4^{o}C. The upper phase of the homogenate was collected into a fresh 0.65mL tube. 90µL of previously prepared buffer AO1 was added to the bottom phase and it was homogenised for 10 seconds to allow membrane proteins to completely complex with detergent and membrane lipids. It was centrifuged at 200*g* for 1 minute allowing for efficient removal of large protein aggregates. This sample was pipetted into the tube containing the upper phase. The combined phases were centrifuged at 750*g* for 10 minutes. This step allows proteins to complex with detergent and membrane lipids and be pelleted. The supernatant was transferred to a fresh tube, left on ice for 60 minutes, and centrifuged again at 12000*g* for 20 minutes to allow collection of only proteins not associated with the plasma membrane, known as the post-plasma membrane (PPM) proteins. The remainder of the protein-detergent complexed pellet was resuspended with

45µL of cold buffer allowing solubilisation of membrane proteins. This was kept on ice for 60 minutes, centrifuged at 12000*g* for 20 minutes, and the supernatant collected as the plasma membrane (PM) fraction.

1.4.2 Protein quantitation

Protein quantitation was done with a Pierce 660nm assay using the 96-well microplate method. A standard curve of known protein concentrations was prepared as a dilution series from 2000µg/µL to 0µg/uL of bovine serum albumin (BSA). 3 standards were prepared, one for each of the homogenisation buffers as a diluent. 150µL of 660nm buffer was added to each well containing 10µL of standard or sample. The total-protein fluorescence of the samples and standards was measured at 660nm using a microplate reader (Molecular Devices Spectramax 190) that Dr. Rudy Boonstra kindly made available to me.

1.5 SDS-PAGE (Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis)

10% resolving and 4% stacking gels were cast using a 15-well comb and the AA-Hoefer Gel Caster Apparatus (10%; 33% 30%-Acrylamide (37.1:1), 33% Separating gel buffer (1.5 M Tris Cl, 0.4% SDS), 55% ddH_2O, 0.65% ammonium persulfate (APS), 5.5% TEMED), (4%; 13.4% 30%-Acrylamide, 9.3% Stacking gel buffer (0.5 M Tris Cl, 0.4% SDS), 33% ddH_2O, 0.06% APS, 3.3% TEMED). Samples were incubated in a 1:1 (w/v) ratio of 2x sample buffer (0.2M DTT, BioRad Laemmli Sample Buffer #1610737) at room temperature for 20 minutes to avoid previously observed aggregation of membrane proteins. The AA-Hoefer SE600 Vertical Gel Electrophoresis apparatus was set up with 6L running buffer (10% BioRad 10X Tris/Glycine/SDS #1610732, 90% ddH_2O). 5µg of sample was loaded into each well, with some wells containing a visible protein ladder (Sigma 26616). The gel was run at 90V for the first 20 minutes and 110V for another 75 minutes with power supplied from an AA-Hoefer PS200HC Power Unit.

1.5.1 Electroblot and immunoblot

SDS-PAGE gel transferred to 0.45µm pore nitrocellulose (NC) membrane (GE Life Sciences #10600003 Protran Premium 0.45 NC) using the AA-Hoefer TE22 Mighty Small Transfer unit at

110V for 90 minutes. The transfer buffer (192mM glycine, 24.8mM Tris, 0.00031% SDS, 20% methanol) contained a proportion of methanol and a very small quantity of SDS to facilitate migration of proteins to the membrane. Following transfer, the membrane was stained with SYPRO Ruby Red Blot (BioRad #1703127) in the following manner: 15-minute membrane incubation in 7% acetic acid/10% methanol solution, followed by 4 × 5-minute ddH_2O washes. The membranes are then incubated with SYPRO stain for 15 minutes in darkness, followed by 4 × 1-minute ddH_2O destaining washes. This destaining was monitored using a UV-light emitter kindly lent to me by Dr. Mauricio Terebiznik. Immediately following staining, the blots were imaged on a Bio-Rad PharosFX Molecular Imager (#1709460). The 532nm laser was used fluoresce the SYPRO dye and the resultant emittance was captured using a 600-630nm band pass filter.

The NC membrane was blocked in 5% BSA in 1X PBST (phosphate-buffered saline, 0.1% Tween-20) for 1-hour during which time 95% of the SYPRO stain was removed. The membranes were then incubated with the appropriate anti-GLUT primary antibody at the following dilutions in PBST buffer: GLUT1 (1:250), GLUT2 (1:2000), GLUT3 (1:2000), GLUT5 (1:500). These concentrations were optimised for 5µg of total protein homogenate per lane (data not shown). The primary antibody was incubated at 4^{O}C overnight with gentle agitation. The use of the cold room was kindly provided by Dr. Ian Brown and Cathy Deane. Membranes were washed 4 × 5-minutes using PBST buffer and then incubated in anti-rabbit horseradish-peroxidase-conjugated secondary antibody (Cell Signalling Technology #7074) at 1:1000 dilution with PBST buffer for 90 minutes with gentle agitation. The membranes were again washed (4 times for 5 minutes in PBST) of remaining unbound antibody. They were incubated for 30 seconds with Pierce Electrochemiluminescent Reagent (Pierce 32106) and imaged using a BioRad Chemidock XRS+ Gel Imager.

1.5.2 PM Fractionation Control and Purity Tests

To quantify the separation of the PM from the cytosolic proteins, antibodies targeting known PM-residing and cytosol-residing proteins were used to probe the fractionated flight muscle and liver samples. To maximise the quantity of data from a single sample, Western blots of GLUT proteins were stripped and reprobed with the antibodies for the control protein. Following visualisation, membrane was stripped using antibody stripping buffer (Gene BioApplication

L.T.D., Kfar-Hanagid, Israel) 2 × 15 minutes followed by 4 × 5 minutes PBST washes. The membrane was blocked again as above and incubated with the appropriate plasma-membrane or cytosolic protein antibody to determine purity of each fraction. The antibodies were incubated at 1:1000 dilution for 90 minutes and included 1) E-cadherin (Cell Signalling Tech. 24E10), 2) Na^+/K^+ ATPase (Cell Signalling Tech. 3010), 3) Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Cell Signalling Tech. 14C10), and 4) Fatty acid translocase (FAT) (Abgent AP2883c). The membrane was washed with PBST 4 × 5 minutes and incubated with the same secondary antibody and steps as above. The membrane was the incubated in Pierce ECL and imaged as described above.

1.6 Normalisation and T-test

The antibody staining intensity of each Western blot sample was normalised to its corresponding total protein stain intensity using BioRad ImageLab software. Background subtraction was applied to the total protein stain in a lane-wise fashion, while no background subtraction was applied to the antibody staining intensity. Lane widths were set to 30% of the lane width for the total protein stain as per the recommendation of Gassmann et al. (2009) while the antibody stain was quantified used fixed-width lanes. To normalise to the total protein concentration, BioRad software was used to divide the intensity of each anti-GLUT band by the integral of the total protein stain intensity. Comparisons of relative abundance of protein-of-interest were then made among gel lanes as well as among blots.

To quantify changes in staining intensity of GLUTs related to treatment (fasted vs. fed), the means of the normalised band intensity from fed and fasted samples were obtained and the ratio of the means were computed using R statistical software. To judge the statistical significance of the relative difference of the fed samples to the fasted, unpaired t-tests were run on the normalised intensities from each treatment using R statistical software. To verify the homogeneity of each treatment group, Levene’s test was performed on fed and fasted samples also using R. Finally, the data was presented as the difference in the mean intensity of the fed treatments to the fasted treatment along with the coefficient of variation. Data was also output as boxplots representing the mean, first and third quartiles of the band intensity from fed and fasted treatments.

2. Results

2.1 Custom GLUT antibody - synthesis and performance

Custom polyclonal antibodies were delivered after affinity-purification of the pooled rabbit serum. The concentration of peptide-binding protein was determined through ELISA by the manufacturer (Table 1). Custom antibodies showed differing reliability and quality. Anti-GLUT1 and GLUT3 showed consistent, tissue specific banding that contained little-to-no nonspecific bands. Anti-GLUT2 and GLUT5, on the other hand, showed multiple non-specific bands in their cytosolic domain; although the band matching the molecular weight of the protein of interest (POI) was the brightest in all blots observed. Furthermore, anti-GLUT2 and 5 showed markedly less nonspecificity in the PM-domain. This suggests that while anti GLUT1 and GLUT3 antibodies are the most reliable tested, GLUT5 and GLUT2 are still informative about the abundance of the POI in the PM-domain.

Table 1: Anti-GLUT 1, 2, 3, and 5 antibodies.

GLUT	Rabbit	Amino-acid sequence	Antibody Conc..
1	PAC11373	Cys-SGFRQGGAGQSDKTPDEFHS	1.1 $mg \cdot ml^{-1}$
	PAC11374		
2	PAC11375	Cys-FRRRKLPTKAMTELEDLRGREEA	5.7 $mg \cdot ml^{-1}$
	PAC11376		
3	PAC11377	Cys-SRGFEGRGDASSPSPVEKVE	2.6 $mg \cdot ml^{-1}$
	PAC11378		
5	PAC11379	KLKGKKHESSDNNDGSK-Cys	1.0 $mg \cdot ml^{-1}$
	PAC11380		

2.2 Ruby-throated hummingbirds

All hummingbirds used in this study were tested within 6 months of initial capture. All hummingbirds appeared healthy and in a normal range of mass at death (average fed hummingbird mass = 3.77g ± 0.71) (average fasted hummingbird mass = 3.51g ± 0.55). Flight muscle for hummingbird H7 (housing number 85) displayed a dull colour and extremely low protein concentrations upon quantitation and was ultimately not included in the study.

Table 2: Ruby-throated hummingbird study reference numbers.

Housing number	Study Ref. Num.	Treatment	Mass at death
70	H1	Fed	N/A
97	H2	Fasted	3.10g
87	H3	Fed	3.02g
76	H4	Fasted	2.56g
95	H5	Fed	5.02g
86	H6	Fasted	3.65g
85	H7	Fed	3.54g
102	H8	Fasted	3.60g
101	H9	Fed	4.40g
98	H10	Fasted	3.93g
96	H11	Fed	3.46g
99	H12	Fasted	4.23g
100	H13	Fed	3.16g

2.3 Total protein control

The total-protein stain was assessed for its strength in indicating the actual total-protein concentration. 1µg/µL muscle homogenate was serially diluted (1µg/µL, 0.5µg/µL, 0.25µg/µL) in SDS-PAGE lanes and subsequently stained with SYPRO Ruby Red Total Protein stain (Figure 1). SYPRO demonstrated an $r^2 = 0.9996$ when plotted against the total protein concentration as measured by the Pierce 660nm assay using the microplate method. This indicates that SYPRO intensity rises linearly with total protein concentration, determining it to be a good indicator of total-protein concentration. SYPRO stain intensity was used in this study for normalisation purposes.

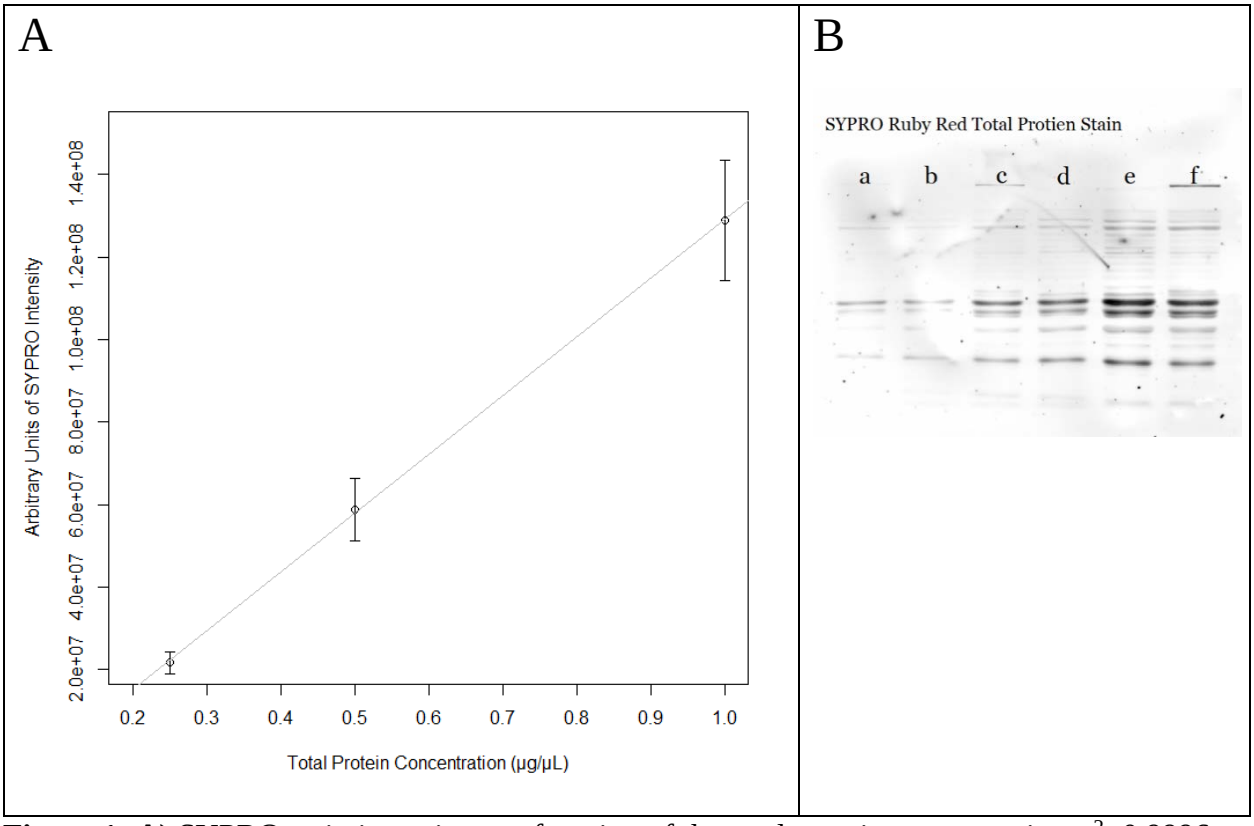


Figure 1: A) SYPRO stain intensity as a function of the total protein concentration. r^2=0.9996. **B)** Representative NC membrane with post-electroblot and stained with SYPRO ruby red blot stain. 532nm laser at 50% power, 600-630nm band pass.

2.4 Fraction purity

Ruby-throated hummingbird tissue underwent homogenisation and PM-fractionation according to the method described by Yamamoto et al. (2016). While this method was described in mice, it has never been attempted in an avian species. The protocol creates three fractions; the cytosolic (whole-cell) fraction, the plasma membrane (PM)-fraction, and the post-plasma membrane (PPM)-fraction. The cytosolic-fraction is also known as a standard RIPA homogenisation and results in a fraction enriched with cytosolic proteins. The PM-fraction is enriched in PM-associated proteins, while the PPM-fraction with all proteins but ones that are PM-associated. The PPM fraction essentially contains cytosolic proteins, vesicles, mitochondria and some peripheral membrane proteins. As such, SDS-PAGE separation and subsequent immunoblotting with PM-associated or PM-unassociated proteins should reveal the respective purity of the PM or cytosol-enriched fractions.

2.4.1 E-cadherin

E-cadherin is a membrane-residing protein where it plays a role in cell-to-cell adhesion (Cavallaro & Christofori, 2004). It has a predicted molecular weight of ~135kDa. Following SDS-PAGE of PM-fractionated hummingbird tissue samples, I observed bands of ~74.3kDa, a known truncation size of E-cadherin (Masterson & O'Dea, 2007), in the following fractions (by order of greatest abundance); PM > Cytosol > PPM (Figure 2A). The intensity of the bands in the PM fraction made up 92.1 ± 1.8% of the total intensity. The PPM-fraction showed 7.9 ± 1.8% of E-cadherin on average (Figure 2A). These fractionation proportions are for fasted hummingbirds, and fed hummingbirds showed very similar proportions summarised in Table 3.

2.4.2 GAPDH

GAPDH is a cytosolic protein with an important role in the glycolysis of imported hexoses (Barber, Harmer, Coleman, & Clark, 2005). It has an expected molecular weight of ~35.8kDa. I observed bands of ~34.9kDa. The signal had the greatest intensity in the PPM, followed by the cytosol. The PM-fraction had the lowest intensity of GAPDH (5.9 ± 0.5%) (Figure 2B). As GAPDH associates with the PM, a small level of GAPDH signal was expected in the PM-fraction (Table 3).

2.4.3 Na^+/K^+ ATPase

The Na^+/K^+ ATPase is a PM-residing transmembrane solute pump with an expected molecular weight of ~100 kDa (Jorgensen, Håkansson, & Karlish, 2003). I observed bands of ~103.1 kDa of highest intensity in the PM-fraction, followed by the cytosol and then PPM fractions (Figure 2C). Approximately 92.1±0.5% of the PM-residing protein found in the PM and 7.8±0.5% in the PPM.

2.4.4 FAT

FAT (fatty acid translocase; aka CD36) is an essential cytosolic shuttling protein involved in the anabolism and catabolism of fatty acid reserves (McWilliams et al., 2004). It has an expected molecular weight of ~50kDa, but usually appears around 70-80kDa in whole-animal tissue, likely due to post-translational modifications such as glycosylation (Feldhahn et al., 2012). I observed FAT at ~89.5kDa with the PPM showing the highest abundance followed by the cytosol and the PM (Figure 2D). While the PPM indicates degree of fractionation as only 71.8% ± 18.6 of the protein was present in the PM (28.2% ± 18.6), PM-FAT varied in staining intensity amongst fractions with feeding treatment and was ultimately not used as a protein-fraction purity indicator. FAT staining in fed hummingbird fractions was ~30% higher than in samples from fasted hummingbirds

Table 3: Abundance of control proteins in the PM and PPM fractions.

Control Protein	**Treatment**	**Intensity in the PM frac.**	**Intensity in the PPM frac.**
E-Cadherin	Fed	92.1 ± 1.8%	7.87 ± 1.8
	Fast	91.3 ± 2.1%	8.7 ± 2.1
GAPDH	Fed	5.9 ± 0.5%	94.1 ± 0.5
	Fast	10.4 ± 0.9%	89.6 ± 0.9
Na^+/K^+ ATPase (pooled samples)	Fed/Fasted	92.1 ±0.5 %	7.8 ± 0.5 %
FAT	Fed	28.2% ± 18.6	71.8% ± 18.6
	Fast	58.5% ± 25.3	41.5% ± 25.3

A) E-Cadherin; observed molecular weight of ~74.3 kDa. LDR r^2 = 0.990.

B) GAPDH; mW ~34.9kDa. LDR r^2 = 0.996.

C) Na+/K+ ATPase; mW ~103.1 kDa. LDR r^2 = 0.979.

D) FAT; mW ~89.5 kDa, LDR r^2 = 0.979.

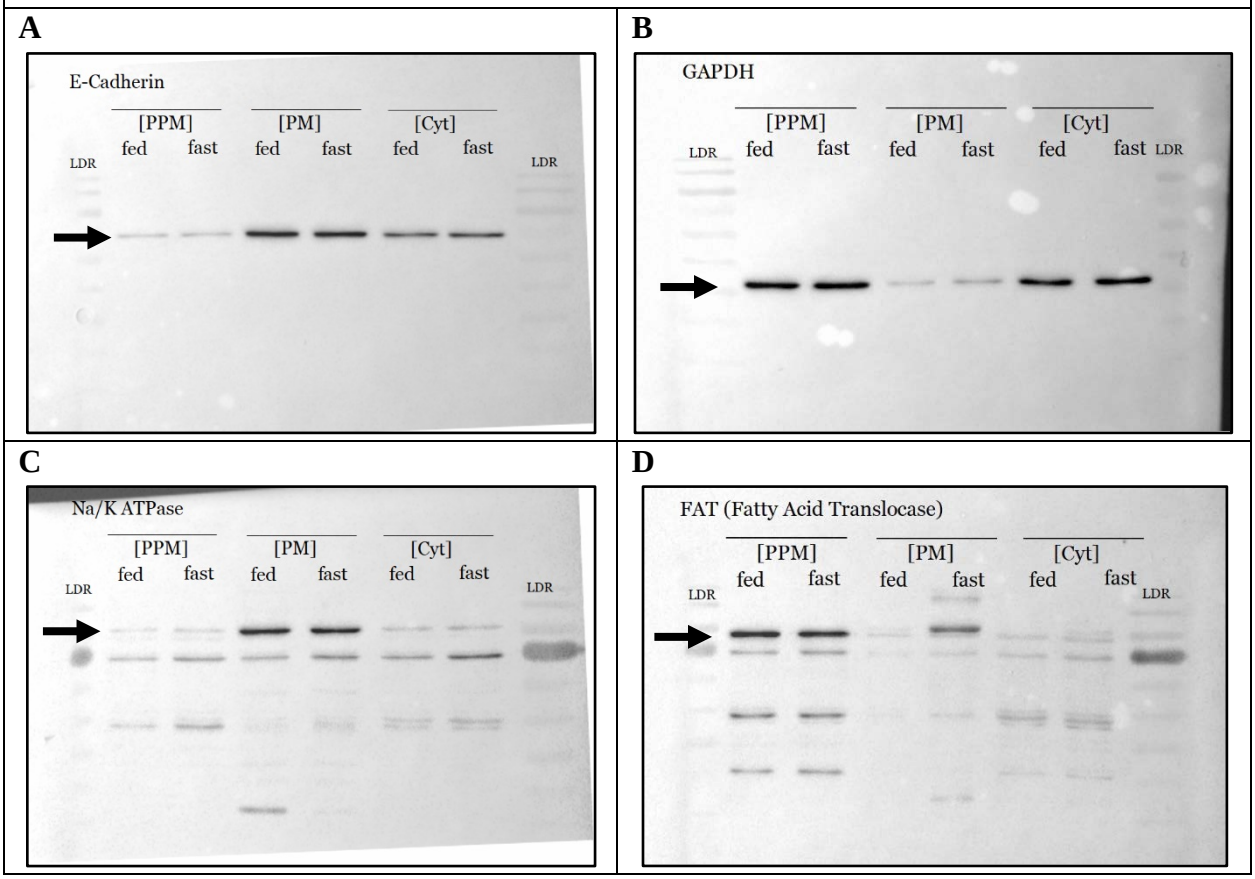


Figure 2: Representative Western blots of E-Cadherin in fed and fasted ruby-throated hummingbird pectoralis tissue superimposed onto a colorimetric image of the visible protein ladder (PL; Thermofisher 26616). Samples include cytosolic RIPA fraction ("Cyt"), plasma-membrane protein fraction ("PM"), post-plasma membrane protein fraction ("PPM"). This fraction contains cytosolic proteins, vesicles, mitochondria and some peripheral membrane proteins. LDR represents the visible protein ladder, while "fed" and "fast" refer to the individual treatments. The black arrow indicates the band-of-interest. All other bands are ghost-bands from a previous immunoblot that was stripped.

2.5 Expression and response to feeding of GLUTs 1, 2, 3, and 5 in hummingbird flight muscle and liver

2.5.1 GLUT1

GLUT1 protein was detected in flight muscles of ruby-throated hummingbirds (Figure 5A), but not the liver (Figure 6A). Muscle anti-GLUT1 bands (observed mW = ~47kDa) were observed in both PM and cytosolic fractions. GLUT1 staining intensity showed the greatest significant increase in the PM fraction (PM: +66%, n=6, p=0.016) and no change in the cytosolic fraction (Cyt: +33% n=6, p=0.133) of flight muscle of recently fed hummingbirds compared to hummingbirds that were fasted for 1 hour (Figure 3). In the liver, GLUT1 protein showed a very weak signal in both the PM and cell fractions; the signal was too faint to confidently obtain the relative protein abundance. As such, it was determined that GLUT1 protein may have little-to-no presence in ruby-throated hummingbird hepatocytes. These results are summarised in Table 4 and representative blots are shown in Figure 3 for flight muscle and in Figure 4 for liver samples.

2.5.2 GLUT2

GLUT2 was detected in the flight muscle (Figure 5B) and liver (Figure 6B) of ruby-throated hummingbirds. Anti-GLUT2 bands were observed in both the PM and cytosolic fractions (observed mW ~50.3 kDa). In the flight muscle, GLUT2 did not show a difference in staining intensity between treatments in the PM or cytosol of flight muscle (PM: +15%, n=4, p=0.723; Cyt: +95%, n=4, p=0.082). In the liver, GLUT2 did not change in the PM fraction (PM: +4% n=3, p=0.701), but was significantly higher in the cell fraction (Cyt: +78%, n=3, p=0.003) in fed hummingbirds in compared to fasted.

2.5.3 GLUT3

GLUT3 was detected in the flight muscle (Figure 5C) and liver (Figure 6C) of ruby-throated hummingbirds. Anti-GLUT3 bands were observed in both the PM and cytosol of flight muscle and liver (observed mW = 72.4kDa). GLUT3 did not show a difference in staining intensity in the flight muscle PM in response to feeding (PM: +17%, n=4, p=0.463), but did so in the cytosolic fraction (Cyt: +41%, n=4, p=0.004). In the liver, GLUT3 showed a significant increase

in the PM (+73%, n=3, p=0.033) but not change in the cytosol (+17%, n=3, p=0.096) in fed hummingbirds compared to fasted

2.5.4 GLUT5

GLUT5 was detected in flight muscle (Figure 5D) and liver (Figure 6D) of ruby-throated hummingbird. Anti-GLUT5 bands were observed almost entirely in the PM (observed mW = 55.3kDa), with little intensity in the cytosolic fraction. Staining intensity in the PM for GLUT5 did not show any change in flight muscle of fed birds compared to fasted (PM: -16%, n=4, p=0.328; Cyt: -30%, n=4, p=0.560). In the liver, GLUT5 was significantly lower in the PM (-21%, n=3, p=-.038), but not the cytosol (+71%, n=3, p=0.082) of fed hummingbirds compared to fasted.

Table 4: Relative mean difference in Western blot GLUT-isoform staining intensity of fed hummingbirds compared to fasted ones. “PM” refers to plasma-membrane fraction samples, while “Cytosol” referes to cytoslic fraction samples.

Tissue	**GLUT**	**Fraction**	**Relative difference** (staining intensity enrichment in fed treatment)	**Unpaired t-test p-value** (* indicates significance)	**n** (unpaired comparisons)
Flight Muscle	1	PM	+66%	0.016*	6
		Cytosol	+33%	0.113	6
	2	PM	+15%	0.723	4
		Cytosol	+94%	0.082	4
	3	PM	+17%	0.463	4
		Cytosol	+41%	0.004*	4
	5	PM	-16%	0.328	4
		Cytosol	-30%	0.560	4
Liver	1	PM	N.S.	N/A	N/A
		Cytosol	N.S.	N/A	N/A
	2	PM	+4%	0.701	3
		Cytosol	+78%	0.003*	3
	3	PM	+73%	0.033*	3
		Cytosol	+17%	0.096	3
	5	PM	-21%	0.038*	3
		Cytosol	+71%	0.082	3

Table 5: Absolute intensity of Western blot GLUT isoform staining intensity normalised to SYPRO total protein stain with background correction applied to each lane. P-values are from Levene's test of homogneity, with **" * "** indicating <0.05 which rejects the hypothesis of equally distributed treatment groups. "PM" refers to plasma-membrane fraction samples while "Cytosol" refers to cytosolic fraction sample.

Tissue	GLUT	Fraction	**Fed Intensity** (Normalised values with coefficient of variation)	**Fast Intensity** (Normalised values with coefficient of variation)	**p-value** (Levene's test)	**n**
Flight Muscle	1	PM	$5.61x10^7$ ± 31.1%	$3.37x10^7$ ± 22.0%	0.128	6
		Cytosol	$7.12x10^7$ ± 22.5%	$5.35x10^7$ ± 35.7%	0.543	6
	2	PM	$8.60x10^7$ ± 42.8%	$7.48x10^7$ ± 63.9%	0.607	4
		Cytosol	$8.02x10^7$ ± 44.9%	$4.13x10^7$ ± 23.5%	0.135	4
	3	PM	$3.19x10^7$ ± 36.1%	$2.72x10^7$ ± 12.7%	0.072	4
		Cytosol	$4.17x10^7$ ± 10.6%	$2.95x10^7$ ± 14.3%	0.703	4
	5	PM	$5.66x10^7$ ± 15.6%	$6.74x10^7$ ± 27.2%	0.262	4
		Cytosol	$4.38x10^7$ ± 37.5%	$6.29x10^7$ ± 95.3%	0.106	4
Liver	1	PM	N.S.	N.S.	N/A	N/A
		Cytosol	N.S.	N.S.	N/A	N/A
	2	PM	$1.30x10^8$ ± 10.9%	$1.25x10^8$ ± 11.0%	0.913	3
		Cytosol	$1.06x10^8$ ± 11.1%	$5.96x10^7$ ± 8.55%	0.315	3
	3	PM	$6.49x10^7$ ± 19.6%	$3.76x10^7$± 20%	0.337	3
		Cytosol	$6.57x10^7$ ± 9.78%	$5.60x10^7$ ± 7.64%	0.392	3
	5	PM	$5.17x10^7$ ± 14.2%	$6.55x10^7$ ± 4.42%	0.093	3
		Cytosol	$4.78x10^7$ ± 20.4%	$2.80x10^7$ ± 39.9%	0.908	3

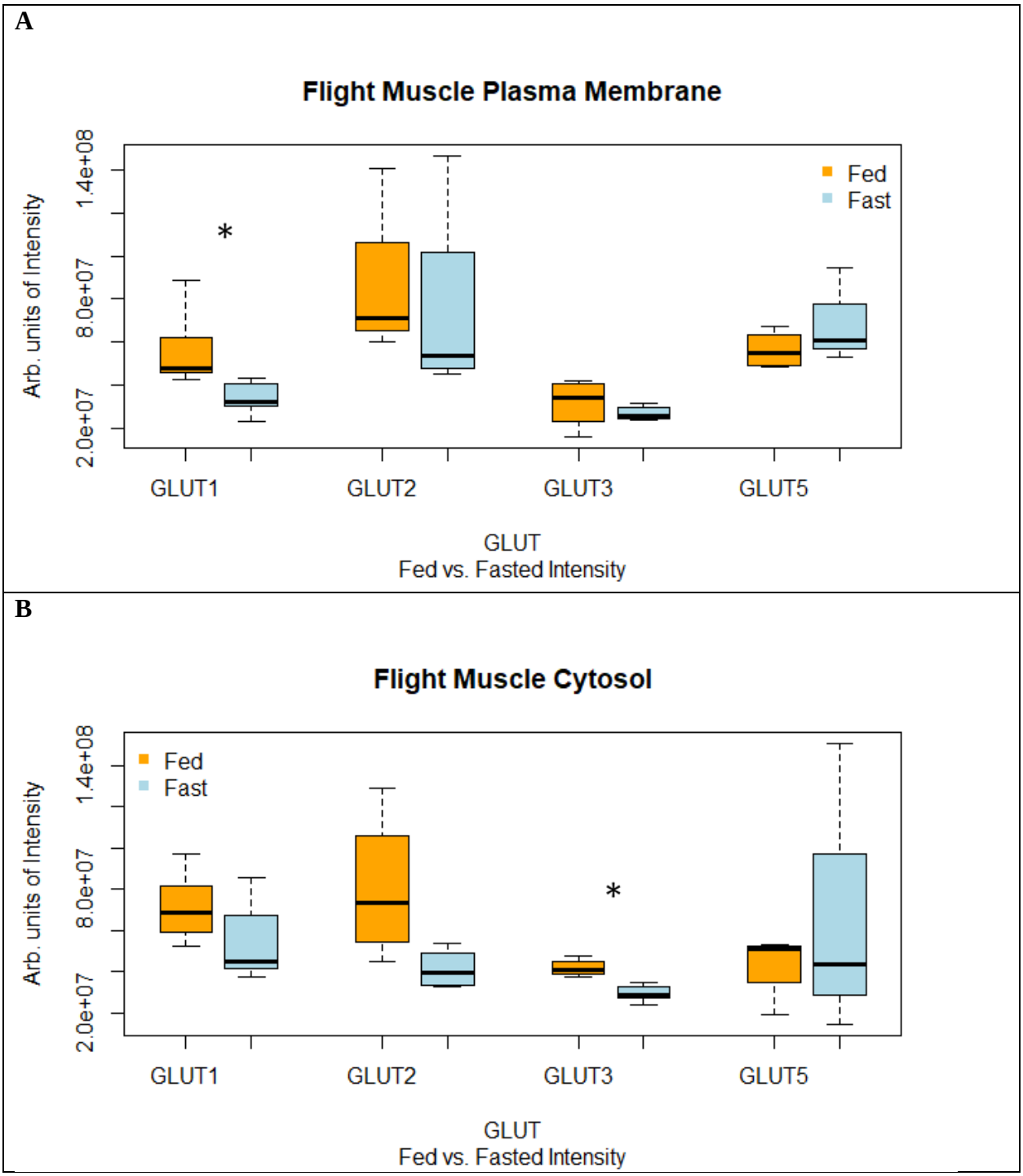


Figure 3: Summary of the flight muscle staining intensities of anti-GLUT-isoforms in fed compared to fasted individuals. Orange indicates the staining intensity in the fed individuals relative to that in the PM in fasted birds (blue). The **" * "** indicates differences between treatments are significant ($P<0.05$). **A)** Relative staining intensity of plasma membrane (PM) fractions. **B)** Relative staining intensity of cytosolic fractions. Statistical data are presented in Table 4 and Table 5. Representative blots are presented in Figure 5.

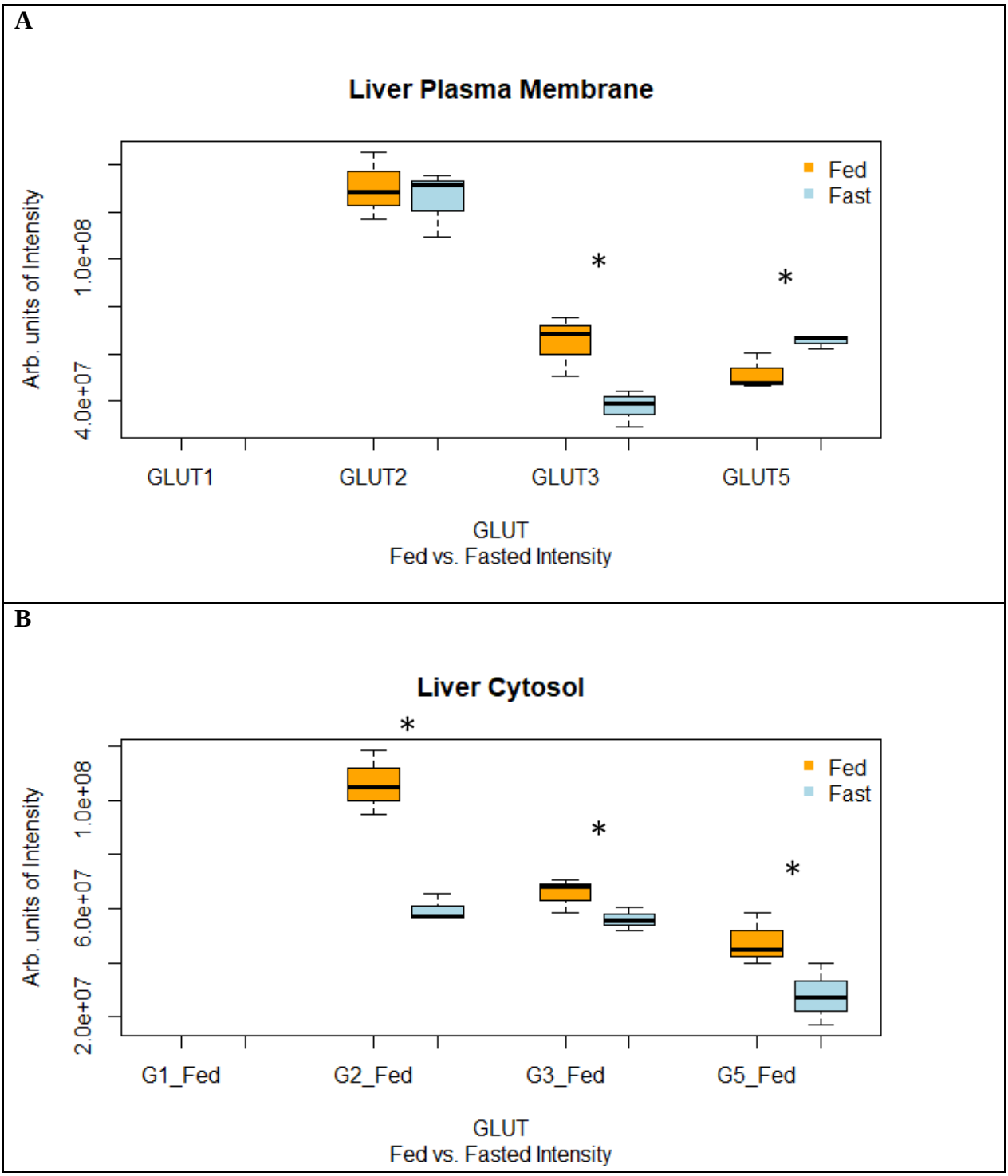


Figure 4: Summary of the liver staining intensities of anti-GLUT-isoforms in fed compared to fasted individuals. Orange indicates the staining intensity in the fed individuals relative to that in the PM in fasted birds (blue). The **" * "** indicates differences between treatments are significant ($P<0.05$). **A)** Relative staining intensity of plasma membrane (PM) fractions. **B)** Relative staining intensity of cytosolic fractions. Statistical data are presented in Table 4 and Table 5. Representative blots are presented in Figure 6.

A) GLUT1; mW ~47.0 kDa. Protein ladder r^2 = 0. 995.
B) GLUT2; mW ~43.5 kDa. PL r^2 = 0.998.
C) GLUT3; mW ~72.4 kDa. PL r^2 = 0.991.
D) GLUT5; mW ~55.3 kDa, PL r^2 = 0.993.

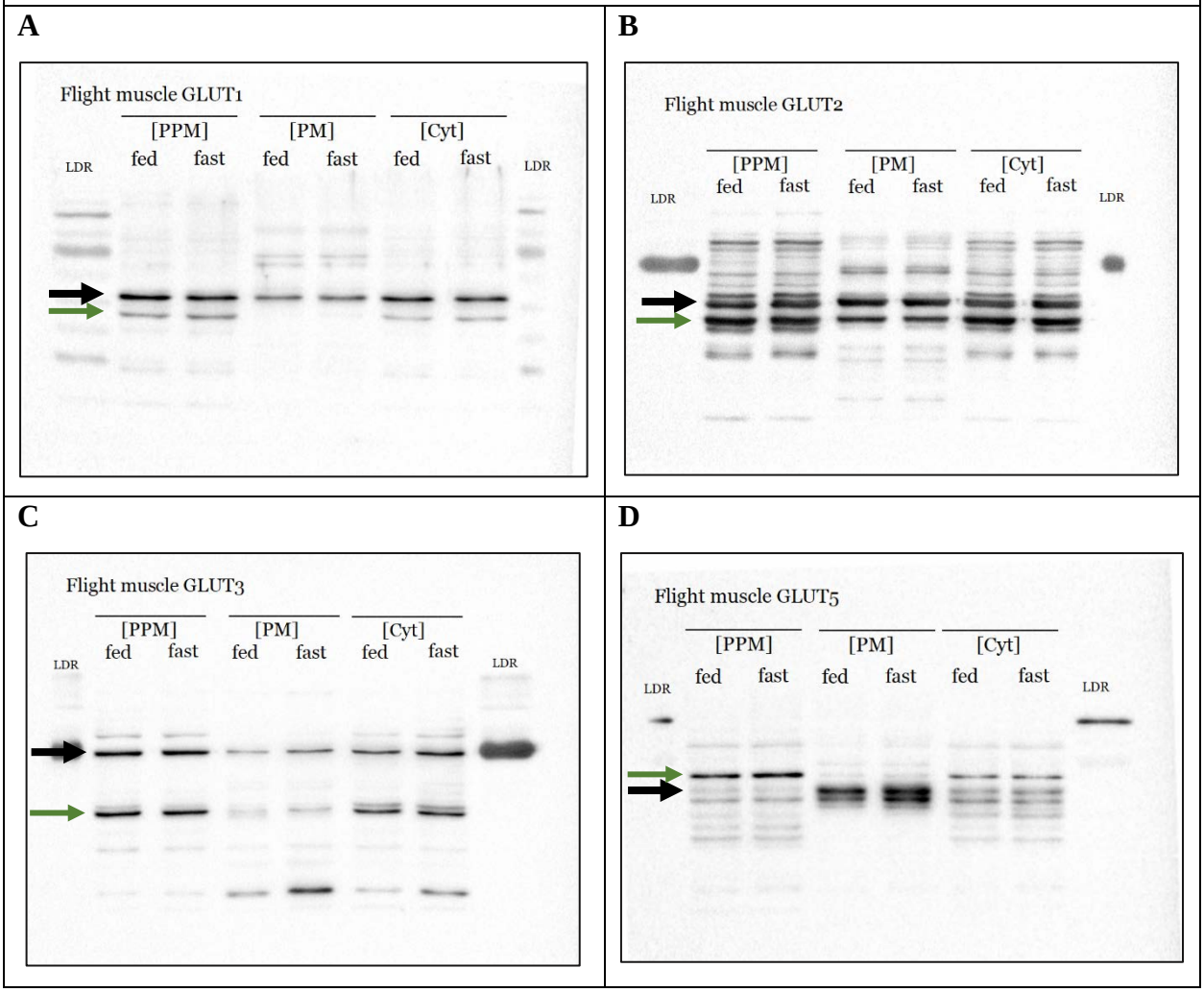


Figure 5: Representative Western blots of GLUT isoforms in ruby-throated hummingbird flight muscle tissue. Samples include cytosolic RIPA fraction ("Cyt"), plasma-membrane protein fraction ("PM"), post-plasma membrane protein fraction ("PPM"). LDR represents the visible protein ladder, while "fed" and "fast" refer to the individual treatments. The black arrow indicates the band-of-interest. The green arrow indicates non-specific banding for that antibody in that particular tissue.

A) GLUT1; N/A. PL r^2 = 0. 9757
B) GLUT2; mW ~43.5 kDa. PL r^2 = 0.998.
C) GLUT3; mW ~72.4 kDa. PL r^2 = 0.997.
D) GLUT5; mW ~55.3 kDa, PL r^2 = 0.998.

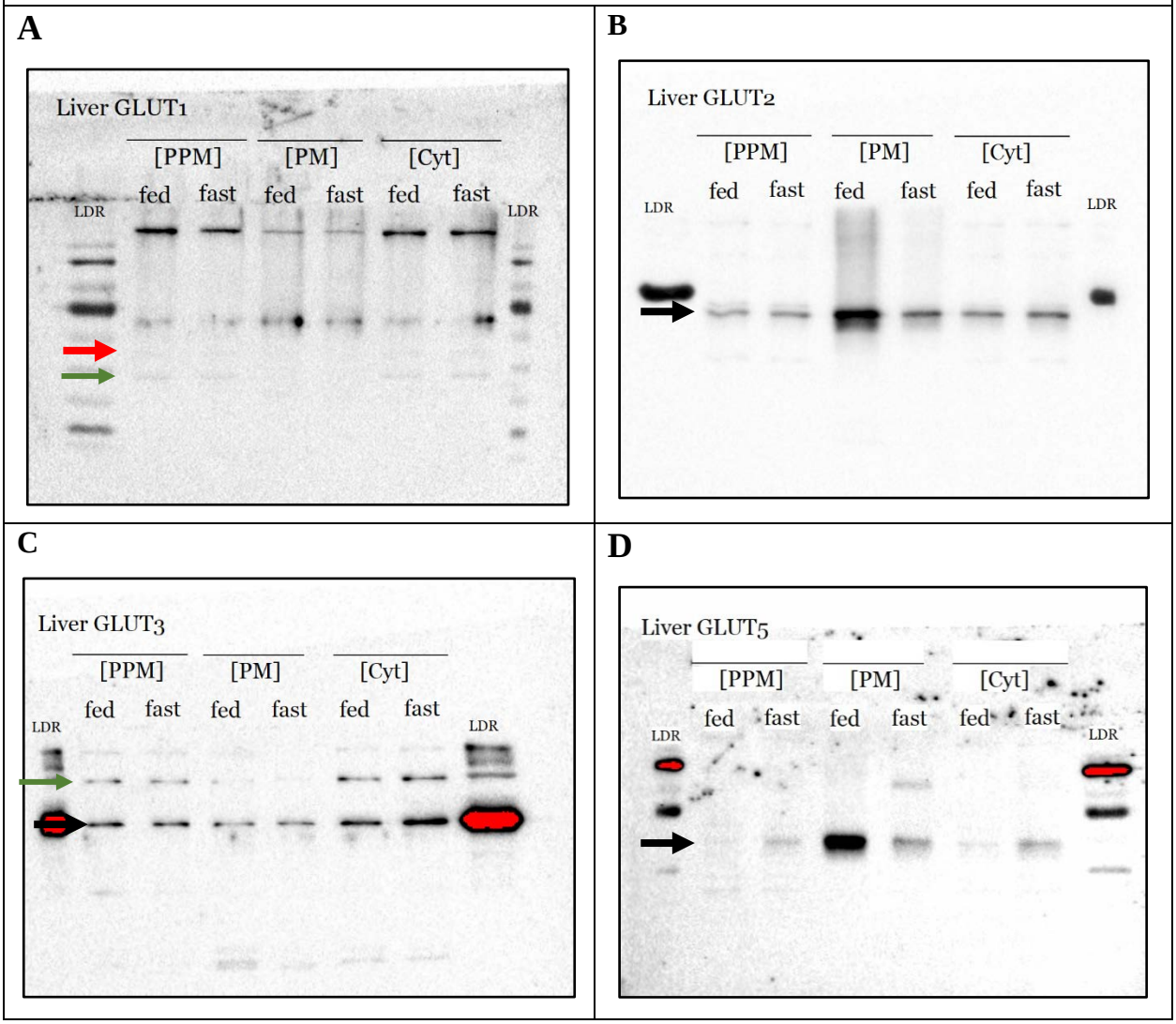


Figure 6: Representative Western blots of GLUT isoforms in ruby-throated hummingbird liver tissue. Samples include cytosolic RIPA fraction ("Cytosol"), plasma-membrane protein fraction ("PM"), post-plasma membrane protein fraction ("PPM"). LDR represents the visible protein ladder, while "fed" and "fast" refer to the individual treatments. The black arrow indicates the band-of-interest. The green arrow indicates non-specific banding for that antibody in that particular tissue. The red arrow indicates the lack of an otherwise expected protein for that tissue.

Chapter 3

Discussion

1. Study design.

1.1 Modification of fractionation protocol.

Glucose transporters (GLUTs) are a family of glycosylated transmembrane transporter proteins that allow transport hexoses through the plasma membrane (PM) of nearly all eukaryotic cells (Thorens & Mueckler, 2010; Uldry & Thorens, 2004). They exist and function in the PM as a family of isoforms, each with its own preferred substrate, kinetics, and tissue-specific distribution (Thorens & Mueckler, 2010). Their location is critical to their function and their abundance in the PM and cytosolic domains may reflect regulation of their activity (Cheeseman & Long, 2015).

Since as early as the 1960s, it has been possible to separate the PM of eukaryotic cells from their cytoplasmic domains (De Duve & Beaufay, 1981; Neville, 1960). This allows observation of the subcellular distribution of these proteins and any translocation that may occur between the domains. I modified a plasma membrane fractionation protocol by Yamamoto et al. (2016) to separate the PM and cytosolic domains of the flight muscle and liver of ruby-throated hummingbirds, *Archilochus colubris*. I then separated the fractions by SDS-PAGE and subsequently Western blotted with custom anti-GLUT 1, 2, 3, and 5 antibodies. 5µg of the PM and the cytosolic fractions were loaded on to SDS-PAGE and normalised to their total-protein stain, allowing any differences in Western blot antibody fluorescence to reflect the relative abundance of the protein-of-interest.

1.2 Custom antibodies: caveats and controls.

Probing tissue using antibody-based techniques has long been a powerful tool in the molecular biologist's repertoire. Recently, it has become relatively affordable for the oft-underfunded non-model organism researcher to relatively easily and cheaply obtain antibodies with a custom epitope for a protein-of-interest. Unfortunately, due to the nature of the market supply-and-demand forces that are beyond the scope of this study, it is in the antibody producers best economic interest that the researcher shares in the bulk of epitope validation.

Antibody epitope specificity is necessary for its primary function; identification of antigens. Polyclonal antibodies are synthesised from a common precursor, with each descendant showing a slight variation in its epitope-recognition of the antigen. As such, I had received 4 antibodies from 8 rabbits targeting 4 very similar transmembrane transporters and it was my job to make sure they bind to what's on their label.

Antibodies may be validated if their application in multiple proteomic techniques yields results expected by the protein-of-interest (POI) (Bordeaux et al., 2010). I chose Western blotting (WB) as my application for antibody validation. I intended to use WB procedures to ascertain the POI's molecular weight along with the manufacture's affinity-purified antibodies. I had initially received promising data, however, as I continued expanding my sample size, the WB data began to appear more random. At this point, I was encouraged by Dr. Mauricio Terebiznik to more thoroughly analyse the SDS-PAGE itself. I observed ~50% of the anti-GLUT staining to be in the stacking layer; this was true for the other GLUT antibodies as well as my control proteins for which I was using commercially available (and validated) antibodies.

I suspected the homogenisation step was leading to protein aggregation as thus staining intensity in the stacking layer. The muscle contractile machinery from my homogenisation method may have been clogging up the pores of my gel, trapping a sizable and random proportion of my POI (Fritz, Swartz, & Greaser, 1989). In February 2018, I obtained a protocol for tissue-based PM-fractionation by Yamamoto et al. (2016). This method excluded the use of complicated ultracentrifugation and sucrose gradients that are involved in the traditional cellular fractionation methodology. It also added steps of quick initial centrifugation and decanting of the homogenate. This allowed for the efficient removal of large protein aggregates. I performed SDS-page on the separated fractions and subsequent WB using commercially available antibodies for the PM proteins E-cadherin and Na^+/K^+ ATPase, and cytosolic proteins GAPDH and fatty-acid translocase (FAT). These control proteins demonstrated that >90% of the PM-residing proteins were in the PM-fraction (Table 3) based on staining intensity. The low intensity staining in the PPM fraction may result from imperfect fractionation, the presence of immature proteins in the ER (endoplasmic reticulum), or random nonspecific association. I concluded that the PM fraction was enriched in PM-proteins, but not cytosolic proteins and vice-versa for the cytosolic fraction.

I proceeded to measure the reliability of the SYPRO total protein stain in reflecting the total protein concentration of each of these fractions. I determined SYPRO intensity rose linearly (r^2=0.9996) with fraction concentration. I proceeded to immune-staining the samples with the anti-GLUT antibodies, confident that my fractionation method produced repeatable results and that normalisation to the total protein stain was accurate. This allowed me to attribute variation in the anti-GLUT staining intensity to underlying differences in GLUT-isoform abundance.

2. Ruby-throated hummingbird glucose transporters.

2.1 GLUT 1, 2, 3, and 5 expression in flight muscle and liver

In this study, I observed GLUT1, GLUT2, GLUT3, and GLUT5 protein in hummingbird flight muscle PM and cytosol (Figure 5A-D). I also observed GLUT2, GLUT3, and GLUT5, but no clear evidence of GLUT1, protein expression in the hummingbird liver (Figure 6A-D). Previous work on the ruby-throated hummingbird has shown mRNA transcript expression of GLUTs 1, 2, 3, and 5 in flight muscle and liver (Myrka & Welch, 2018; Welch et al., 2013). I observed these isoforms at the protein-level in this study in hummingbird muscle and liver, except for hepatic GLUT1.

GLUT1 protein has previously been detected in the hummingbird flight muscle through staining with a commercially available antibody (Welch et al., 2013). However, this work indicated that the observed molecular weight (mW) was ~70kDa and that GLUT1 was detected in the hummingbird liver (Welch et al., 2013). This is in opposition to my study that found GLUT1 at mW ~47.0 kDa. Furthermore, I did not detect GLUT1 protein in the liver using custom polyclonal antibodies (Figure 4A) targeting the ruby-throated hummingbird sequence for GLUT1. The discrepancy in the band size, particularly the detection in the liver may have been artefactual or represented non-specific binding in the previous study. The highly-glycosylated nature of GLUT1 protein (Samih et al., 2003) may also underlie these results; Western blots of mammalian myocytes shows GLUT1 protein expression, but with smearing from ~50-100 kDa, reflecting the variety in possible glycans (Mitsumoto & Klip, 1992). As such, the single ~47.0 kDa GLUT1 band I observed in this study may be reflective of differential glycosylation in the hummingbird; with only a single variant of GLUT1 glycan present on the protein. GLUT1 protein in the avian liver is largely uncharacterised as most studies focus on the presence of

mRNA transcript (Wagstaff & White, 1995, p. 1). However, work on chicken liver has also demonstrated missing GLUT1 protein despite presence of mRNA transcript (Byers et al., 2018, 2017; Carver et al., 2001), which may have resulted from contamination of erythrocytes which are known to have a large abundance of GLUT1 (Boulter & Wang, 2001) This study matches these observations of GLUT1 protein presence in the hummingbird muscle and liver.

GLUT2 mRNA transcript is highly expressed in hepatocytes in mammals and birds alike (Kayano & Bell, 1988; Zhang, Sumners, Siegel, Cline, & Gilbert, 2013) and is the primary hepatic transporter involved in hexose uptake in fed and fasted states (Thorens & Mueckler, 2010). GLUT2 protein is also highly expressed in rat liver and its PM abundance shows sensitivity to variation in blood glucose concentrations (Burcelin & Eddouks, 1992). GLUT2 mRNA expression in skeletal muscle was found to be higher than that of the liver in human tissue (McCulloch et al., 2011). Previous work has also found GLUT2 mRNA transcript in ruby-throated hummingbird liver (Welch et al., 2013; Workman et al., 2018). In this study, I observed GLUT2 protein expression in both the muscle and liver of ruby-throated hummingbirds. The observed anti-GLUT2 band, mW ~50.3 kDa is close to the predicted weight of GLUT2 at 53 kDa.

I observed GLUT3 in both the PM and cytosol of hummingbird flight muscle and liver. Staining intensity for GLUT3 was greater in the cytosol of these tissue compared to their PM-fraction. My results are in partial agreement with studies on rat PC12 cells; a high degree of GLUT3 staining was detected in the cytosol (Thoidis et al., 1999; Thorens & Cheng, 1990), although the PM:Cytosol ratio was smaller than what I observed in hummingbirds. I observed GLUT3 at mW ~72.4kDa. This is in contrast to previously-found GLUT3 protein in chickens that is ~45kDa (Wagstaff & White, 1995). However, this may also be due to variation in GLUT3 glycosylation as observations of GLUT3 protein in Chinese hamster ovary cells showed a broad band of 45-65kDa attributable to intensive glycosylation of the protein (Asano et al., 1992).

GLUT5 protein is expressed at very low levels in mammalian skeletal muscle (Kayano & Bell, 1990), reflecting the reduced role of fructose in muscle metabolism. Hummingbirds show expression of GLUT5 mRNA transcript in muscle (Welch et al., 2013) as well as a relatively high fructose-phosphorylation capacity (Myrka & Welch, 2018). My results confirm the expression of GLUT5 isoform in hummingbird flight muscle; a crucial transporter facilitating the

unique possible ability of hummingbird muscle to directly oxidize fructose. It is interesting to note that very few studies detect GLUT5 in avian muscles, and the isoform is often excluded from study (Byers et al., 2018; Sweazea & Braun, 2006). One possible reason for this is the difficulty of successfully probing for GLUT5, whether it is protein or mRNA transcript, and may arise as GLUT5 protein was the earliest to diverge away from early eukaryotic GLUT1 (Wagstaff & White, 1995). Indeed, GLUT5 shows ~68% homology between hummingbirds and humans (Appendix 2). If chickens also show similarly low GLUT5 abundance in their muscle, it would be apparent that hummingbirds are unique, even amongst birds, in their muscle fructose-oxidation capacity

Studies on avian-specific subcellular distribution of GLUT proteins are sparse and largely focus on *in vitro* cell lines or chicken, *Gallus gallus*, models (Byers et al., 2017; Kono et al., 2005; Wagstaff & White, 1995). Further, until now, none of these studies has examined the dynamic of GLUT subcellular localisation in relation to feeding state. Thus, the observations presented here both offer the first description of subcellular localisation of GLUTs in hummingbirds and provides critical insight regarding how these dynamically change in response to variation in energy availability.

2.2 GLUT1 may be involved in facilitating glucose uptake in the muscle, but not the liver.

Hummingbirds have a physiology that is heavily adapted for the metabolic catabolism of recently ingested carbohydrates above all other fuels (Suarez et al., 1991; Welch et al., 2006). Hummingbirds rapidly switch from an almost complete reliance on fatty acid metabolism to fuel foraging activity to exclusively with recently ingested carbohydrates within minutes of consuming a nectar meal (Welch et al., 2007). This extreme shift in the makeup of the fuel mixture used to support exercise is unusual among vertebrates as humans and other mammals typically oxidise both endogenous fats as well as endogenous (glycogen) and exogenous (ingested) carbohydrates to fuel a broad range of exercise intensity (McWilliams et al., 2004). Thus, it seems there is robust and exquisite control over flux through the glycolytic and lipolytic pathways in hummingbirds and I predicted I would observe a corresponding robust difference in apparent sugar transport capacity into active tissues, manifested as changes in PM abundances of GLUTs comparing fasted and fed individuals In this study, I observed significantly higher

intensity of the anti-GLUT1 band in the PM-fraction (PM: +66%, n=6, p=0.016) and cytosolic-fraction (Cyt: +33%, n=6, p=0.133) of ruby-throated hummingbird flight muscle from the fed individuals compared to the fasted (Figure 5). I did not observe any significant difference in the relative distribution of flight muscle GLUT2, 3, and 5.

Changes in PM GLUT1 abundance may be involved in the initial switch to carbohydrate metabolism (Suarez et al., 1991; Welch & Suarez, 2007). As avian muscle is largely insulin-insensitive (Chen, 1945; Dupont, 2009), carbohydrates themselves (perhaps glucose specifically) may act as a potent signal; inducing upregulation of GLUT isoforms to the PM of active cells. Combining the observation of high maximal glucose phosphorylation rates of the hummingbird muscle hexokinase (Myrka & Welch, 2018), with the idea that PM-GLUT isoforms rise in conformation with rising blood sugar levels (Wasserman, 2009), suggests that increased recruitment of GLUT1 to the PM may underlie the initial regulatory steps involved in the dynamic upregulation of carbohydrate metabolism as part of the hummingbird sugar oxidation cascade (SOC). The positive shift in the fed:fasted ratio of PM GLUT1 staining intensity is simultaneous with a negative shift in the same ratio in the cytosol fraction (Table 4). This suggests that at least some of the apparent increase in GLUT1 abundance in the PM in fed birds is attributable to translocation from the cytosolic pool, rather than increased expression or translation.

I also observed a lack evidence for PM-recruitment of GLUT2, GLUT3, and GLUT5 in the hummingbird muscle (Figure 3) in conjunction with feeding. Before assuming a reduced role of these isoforms, a few points must be considered. Firstly, from a mammalian perspective, muscle GLUT2 only rises when the extracellular glucose concentration reaches 30-50mM (Helliwell et al., 2000). As mammalian blood is not typically this hyperglycaemic, ranging from 5-6mM in healthy humans (Zimmet, Magliano, Herman, & Shaw, 2014), GLUT2 is generally not observed to increase in abundance the PM following feeding. In mammals, though both proteins can be detected, neither GLUT2 nor GLUT5 protein is not found in high abundance in mammalian muscle (Thorens & Mueckler, 2010) and neither show recruitment to the muscle PM in relation with feeding status or glucose availability (Helliwell et al., 2000). Secondly, hummingbirds in this study were sedentary and not utilising the full aerobic capacity of their muscles, which can equates a 10-fold increase in metabolic activity (Suarez, 1992). As such, the flight muscles of

these rested hummingbirds may not require such high rates of hexose import, with the increase in PM GLUT1 enough contribute to basal hexose uptake and possibly to prime the cells for future carbohydrate delivery.

2.2.1 Hummingbird hepatocytes do not express any GLUT1 protein.

In vertebrates, energy in the form of circulating hexoses is usually transient; dictated largely by dietary state (Nicolson & Fleming, 2003). As glycaemic regulation is tissue-dependant (Klip & Vranic, 2006), it may be assumed that GLUTs coordinate their primary function in a manner that is largely dependent on tissue-specific energetic needs. Regulation of the system as a whole may be achieved through a family of diverse and tissue-specific isoforms (Thorens & Mueckler, 2010). Indeed, avian carbohydrate regulation systems show differential shuttling of ingested carbohydrates. In the context of feeding, hummingbirds, being ‘energy-maximizers’, will redirect calories to hepatic *de novo* lipogenesis pathways (Suarez, 1998).

I observed a differing response to feeding of hepatic GLUT isoforms compared to muscle in the ruby-throated hummingbird. The most striking observation is the lack of clear detection of any GLUT1 protein in either the PM or the cytosol of hummingbird hepatocytes. This is in stark contrast to the presence of mRNA transcript for hepatic GLUT1 in hepatocytes, including hummingbirds (Byers et al., 2018; Welch et al., 2013). Few studies attempt to explore the protein abundance of these transporters. I presume this is largely due to a focus on observing the coordinated regulation of multiple GLUT isoforms; a task that is technically feasible using conventional PCR, but not with Western blotting. Furthermore, Western blotting of membrane-residing proteins is difficult due to their tendency to form hetero-oligomers when exposed to heat, even in the presence of denaturants (Fritz et al., 1989). GLUT-proteins show heavy glycosylation which usually exacerbates this issue (Kumagai, Dwyer, & Pardridge, 1994). As such, I report here, in agreement with only a handful of studies (Byers et al., 2017; Carver et al., 2001), that the avian liver does not express GLUT1 protein. As such, hepatic GLUT1 cannot be involved in the switch to primarily carbohydrate metabolism in the hummingbird liver.

3. Hummingbird GLUT regulation.

3.1 Hummingbirds may prioritise hepatic carbohydrate throughput while sedentary.

Hummingbirds are considered “energy maximisers”, carefully using nectar stored in their crop to facilitate foraging while still maintaining rapid gut transit rates for ingested carbohydrates (Diamond, Karasov, Phan, & Carpenter, 1986). They also appear to minimise their energy-expenditure, feeding for no more than 20% of their waking hours, and typically hovering for no more than 10-30 seconds at a time (Diamond et al., 1986; Suarez et al., 1990). Perched hummingbirds feeding on nectar will show RER $\geq$ 1.0, preferring circulating carbohydrates for metabolism over endogenous fatty acid stores (Suarez et al., 1990). Hummingbirds in states of caloric excess show increases in mass, accelerated towards their migratory season, that are largely attributable to increases in adipose stores and may underlie the increase in the RER value past 1.0 (Carpenter et al., 1993; Hou & Welch, 2016). As the avian liver is the primary site of *de novo* lipogenesis (McWilliams et al., 2004), it can be surmised that feeding increases hepatic carbohydrate throughput. Indeed, the hummingbird maximal rates of substrate flux through fatty-acid synthesis and gluconeogenic pathways are higher in liver than in almost any other vertebrate tissues, processes requiring carbohydrate import from facilitated glucose transporters (GLUTs) (Burcelin & Eddouks, 1992; Suarez, Brownsey, Vogl, Brown, & Hochachka, 1988; Suarez et al., 1990).

PM GLUT3 protein levels were significantly more abundant in the liver of fed hummingbirds (+73%, n=3, p=0.033) compared to fasted (Figure 4) while no difference in the PM-abundance of these isoform were seen in the muscle (Figure 3). I also observed significantly greater and higher staining intensity in cytosolic hepatic GLUT2 of fed hummingbirds (Cyt: +78%, n=3, p=0.003), but not in cytosolic hepatic GLUT3 staining (+17%, n=3, p=0.096). GLUT2 and 3 staining in the cytosolic fractions of muscle cells was similar in both treatment groups. This indicates that hepatic GLUT3 is responsive to feeding, showing increased PM abundance with feeding, while flight muscle GLUT3 does not respond.

The upregulation of these particular isoforms is significant as GLUT3 is a high affinity carbohydrate transporter (Thorens & Mueckler, 2010; Uldry & Thorens, 2004; Zhao & Keating,

2007). GLUT2 has an affinity for both glucose and fructose and is known to rise in the PM proportionally to their circulating concentration, showing greater PM in response to increased fructose levels rather than glucose (Helliwell et al., 2000; Kellett et al., 2008). Hepatic GLUT2 is heavily involved in the rapid equilibration of cytosolic and extracellular carbohydrate concentrations in a bidirectional manner (Burcelin & Thorens, 2000; Thorens, 1996; Wood & Trayhurn, 2003). These features, glucose and fructose affinity, high capacity transport, bidirectional movement are typical characteristics of the avian liver and GLUT2 seems exceptionally well-suited to play a dominant role. Indeed, GLUT2 is reported as being the most abundant isoform in chicken hepatocytes (Byers et al., 2018; Kono et al., 2005; Zhang et al., 2013).

GLUT3 has the highest affinity for glucose of all the known GLUT isoforms (K_m glucose = 1.4mM (Zhao & Keating, 2007)). It is also known as the brain-type isoform (Arbuckle, Kane, Porter, Seatter, & Gould, 1996) but has been found in avian liver and other tissue requiring rapid rates of glucose import (Maher & Vannucci, 1993; Vannucci, 2008; Zhang et al., 2013). Its high affinity is thought to allow small increases in PM abundance of GLUT3 to lead to large changes in glucose flux (McMillin, Schmidt, Kahn, & Witczak, 2017; Thorens & Mueckler, 2010), an advantageous feature for an organ as critical as the liver. As well as this, avian liver is missing GLUT1 protein, and hepatic GLUT3 may replace its role in hummingbirds as the primary facilitator of glucose transport, unlike in mammals that do express hepatic GLUT1 (Cheeseman & Long, 2015). Thus, I assumed changes in GLUT3 PM abundance levels reflect changes in the hepatic transport capacity for glucose specifically.

In summary, I observed the upregulation of high-throughput GLUT3 in the PM of hummingbird hepatocytes following feeding. This may reflect increased rates of post-prandial carbohydrate processing by the liver.

3.2 Post-prandial recruitment of hepatic GLUT2 and GLUT3 may result from hyperglycaemia and the insulin-sensitivity of avian liver.

It is interesting to note that hepatic PM GLUT3 was in greater abundance in fed individuals, while flight muscle GLUT3 was not. I only observed higher abundance of GLUT1 protein of

muscle PM (PM: +66%, n=6, p=0.016). The reduced response from other muscle GLUT isoforms may reflect the redirection of carbohydrates towards the liver in times of caloric excess and low energy-expenditure (Carpenter, Paton, & Hixon, 1983; Suarez et al., 1990). Or perhaps the expression of GLUT1 is sufficient to facilitate glucose import in the muscles, but its absence necessitates glucose-specific GLUT3 and glucose-transporting GLUT2 to make up hepatic glucose transport.

But how do the liver and muscle coordinate such disparate relative rates of carbohydrate uptake? Perhaps this is a passive redirection; reduced muscular activity can leave capillary recruitment at a minimum (Clark, Rattigan, Barrett, & Vincent, 2008), reducing transport of carbohydrates to sites of active cells and increasing the energy required to overcome the 2nd barrier in the sugar oxidation cascade (Sové et al., 2017; Suarez & Welch, 2011). The drive for carbohydrate import into muscles may also be reduced via fewer active mitochondria, a type of hypometabolic regulation that is characteristic of hummingbird energy-balance (Calder, 1994; Suarez et al., 1991). This may leave the muscles acting as less of a ‘sink’ for carbohydrates, freeing up a greater proportion for incorporation into hepatic, instead of muscle, metabolic and lipogenic pathways.

GLUT2 PM abundance demonstrates close coordination with local glycaemic levels. In isolated rat jejunum, PM GLUT2 levels did not rise until the extracellular glucose concentration reached ~30-50mM, with the protein abundance doubling as glucose concentrations reached 75-100mM (Kellett et al., 2008). Fed ruby-throated hummingbird are known to reach 42mM circulating glucose (Beuchat & Chong, 1998). If the pattern of hummingbird GLUT2 recruitment is similar that found by Kellet et al. (2008), it can be predicted that GLUT2 abundance should be increase by approximately 10-20% in fed hummingbird compared to fasted. Indeed, in this study I observed no difference in the abundance of both hepatic PM GLUT2 and myocyte PM GLUT2 in fed or fasted individuals, further suggesting that hummingbird GLUT2 may play a greater role in glucosensing, rather than feeding-related increase in transport capacity.

On the other hand, if local glycaemia of rested flight muscles is like that of the liver, this does not fully explain why only hepatic GLUT3 showed an increase in PM abundance. Chickens have shown to release insulin in response to feeding, however, its effects in the avian muscle are largely diminished due to a 30-fold increase in PI3K activity (Dupont, 2009). As such, insulin

cannot mediate GLUT-recruitment in the muscle PM. While the data is still sparse, chicken hepatic GLUT3 abundance shows sensitivity to the administration of exogenous insulin while that of muscle does not (Dupont, 2004). Therefore, the hummingbird hepatic GLUT recruitment pattern may be sensitive to both hyperglycaemia and insulin, while, while the muscle GLUTs are sensitive to only hyperglycaemia.

Successful enlargement of hummingbird lipid-stores, especially towards the migration period, depends on accumulation of excess calories that allow for lipogenesis as well as fueling foraging itself (Carpenter et al., 1983). Insulin-signalling may be crucial in allowing independent communication of organs involved in hummingbird glycaemic flux. This may underlie the apparent diel and seasonal shifts of hummingbird lipogenic capacity. Ruby-throated hummingbirds show a daily decline in mass during the midday period, but do not show this drop during the pre-migratory season where increased foraging effort and hyperphagia facilitates *de novo* lipid synthesis from recently ingested sugars (Hou & Welch, 2016). My observations of increased hepatic, but not muscle, GLUT3 may reflect a concomitant signalling that takes place in the liver, but not the muscle. Due to the insulin-resistant nature of birds, it has often been overlooked as a significant source of glycaemic regulation, especially in hummingbirds. I hypothesize that insulin plays a key role in the liver-specific regulatory response to rising blood sugar levels in hummingbirds. However, this data warrants further studies observing circulating insulin levels in hummingbirds. This is logistically challenging due to the incredibly small volume of blood that necessitates lethal sampling to acquire (~70µL; personal observations). Thus, exogenous administration of insulin and observation of the resulting change in PM GLUT abundance may be needed to observe the tissue-specific regulatory effects of insulin in the hummingbird.

3.3 Muscle GLUT1 and liver GLUT3 show capacity for PM recruitment.

The subcellular location of GLUTs is critical to their transmembrane hexose transport function (Guma et al., 1995). As any other protein, their translation and synthesis occurs in the cellular cytosolic domain, after which they are shuttled into vesicles bound for fusion with the plasma membrane (Guma et al., 1995; Palade, 1975). The fusion of these secretory GLUT vesicles is regulated by the eukaryotic cell, sensitive to a variety of intra- and extracellular factors such as

glycaemia, insulin, exercise, and stress, to name a few (Egert, Nguyen, & Schwaiger, 1999; Guma et al., 1995; Yang & Holman, 1993). GLUT isoforms have shown coordinated responses to these stimuli, showing organism- and tissue-specific recruitment rates to the PM and activation of hexose transport (Byers et al., 2017; Wagstaff & White, 1995). As such, relative the abundance of cytoplasmic GLUT proteins in the ruby-throated hummingbird muscle and liver tissue should correlate with the potential capacity of that tissue for GLUT recruitment to the PM, i.e. the cell's capacity to rapidly increase facilitated transmembrane hexose transport. Furthermore, this capacity should reflect known capacities of the isoforms for their preferred substrate, as there seems to be high degree of conservation of substrate-specificity across vertebrates (Byers et al., 2017; Verdon et al., 2016).

I observed that anti-GLUT1 bands stained with a greater intensity in the cytosolic-fraction than in the PM-fraction of normalised flight muscle fractions from fasted hummingbirds (Figure 5A). I also observed a relatively higher cytosolic band intensity of anti-GLUT3 (mW ~ 72.4 kDa) in the liver Figure 4C, but not the flight muscle (Figure 3C). This may be indicative of substantial, recruitable reserves of GLUT1 and GLUT3 protein in the cytosol. Specifically, GLUT1 and GLUT3 were approximately 2-fold higher in the cytosol of muscle and liver compared to their normalised PM-abundance. My observations of higher GLUT presence in the cytosolic fraction may allude to the capacity of that isoform for PM-recruitment. I conclude that GLUT1 in the flight muscle and GLUT3 in the liver show the clearest evidence of significant translocation to the PM in ruby-throated hummingbirds.

3.3.1 GLUT2 and GLUT5 show low recruitment capacity compared to GLUT1 and GLUT3.

I observed anti-GLUT2 bands of similar intensity in normalised PM and cytosolic fractions (Figure 5B, Figure 6B). While this indicates that there is GLUT2 protein in the cytosol, its abundance relative to PM GLUT2 was much lower than for GLUT1 or GLUT3. Continuing with the previous reasoning, If the cytosolic abundance is indicative of a recruitable population, this indicates that GLUT2 may have less capacity for recruitment to the PM than GLUT1 or GLUT3. These findings match our current understanding of GLUT2. Its recruitment to the PM is tied closely with circulating glycaemic levels conferring it with a gluco-sensing function (Thorens, 1996; Thorens & Mueckler, 2010). As noted previously, while GLUT2 is recruitable with

increasing hexose concentrations, it does not have a very high capacity for increase in overall PM abundance (Kellett et al., 2008).

Anti-GLUT5 staining was almost entirely in the PM, with very little observable in the cytosolic domain of both flight muscle and liver alike. The ratio varied among individuals, but PM GLUT5 staining intensity was 5- to 20-fold greater than in the cytosol of fasted hummingbird flight muscle and liver (Figure 5D, Figure 6D).

These findings are further supported by the PM-recruitment patterns of GLUT-isoforms I observed in this study. Muscle GLUT1 and liver GLUT3 showed the greatest increase in PM abundance associated with feeding (+66% and +73% respectively; Table 4). GLUT2 staining intensity in the PM did not change in association with feeding in muscles or hepatic PM fractions (Table 4). Finally, GLUT5 did not show any change in PM-abundance with feeding in muscle, while showing a significant decrease in liver tissue PM (PM: -21%, n=3, p=0.038) (Table 4). This pattern of GLUT-recruitment mirrors the abundance of the protein in the cytosol compared to the PM of fasted hummingbirds. GLUTs 1 and 3 have the greatest cytosolic abundance while GLUT2 and GLUT5 show much less protein in the cytosol compared to the PM (Figure 5, Figure 6). This indicates that the abundance of cytosolic GLUT protein closely reflects the observed changes in PM-abundance.

It should be noted that the decrease in hepatic PM-GLUT5 abundance does not contradict previous findings of the fructolytic capacity unique to the hummingbird flight muscle system (Chen & Welch, 2014). Fructose transmembrane transport may not require further GLUT5 recruitment. Considering that constitutively expressed GLUTs, if in an active state, allow for the continuous import of hexoses, and that hummingbird fructose-phosphorylation capacity is amongst the highest recorded in vertebrates (Myrka & Welch, 2018), this suggests that hummingbird flight muscle and liver cells maintain a constant non-trivial capacity for fructose uptake. Furthermore, this may indicate an eventual reduction in fructose import as it is rapidly removed from the bloodstream. Indeed, mammalian circulating fructose shows rapid regulation, with circulating concentrations existing only between 0.1 and 1mM (Feinman & Fine, 2013). Despite this rapid regulation, mammalian models also fail to show GLUT5 PM recruitment in enterocytes and hepatocytes and near-zero abundance in the muscle (Helliwell et al., 2000), giving further weight to the idea of its constitutive function presence in the hummingbird.

4. Hexose transport capacity of hummingbird GLUTs.

GLUTs exist as a large family of isoforms each with its preferred hexose substrate and transport kinetics. GLUTs are thought to have evolved from a single common hexose transporter, with the fructose-carrying GLUT5 showing the earliest divergence away from the glucose-specific GLUT1 (Thorens & Mueckler, 2010). GLUT3 represents the most recent divergence from GLUT1 and may have occurred around the time avian and mammalian classes diverged away from each other (Wagstaff & White, 1995). Due to the hyperglycaemic nature and relative insulin-insensitivity of birds compared to mammals, it follows that the avian-specific GLUT regulation may occur in a manner different from that of mammals; reflective of the more extreme glycaemic environment their cells face. A theory of GLUT regulation describes the coordinated function of a basal and a recruitable form for a particular hexose (Fladeby, Skar, & Serck-Hanssen, 2003, p. 3; Wagstaff & White, 1995). This theory further postulates that pairs of GLUT isoforms that transport similar substrate show different recruitment patterns between mammals and birds (Fladeby et al., 2003, p. 1; Helliwell et al., 2000). It should also be noted that transport kinetics of the isoforms themselves may be different between mammalian and avian GLUT-isoforms. As detailed above, GLUT-isoforms also show different tissue expression in hummingbirds when compared to other mammals or even other birds. As such, the pairs that GLUT-isoforms function in may reflect the physiological requirements of the hummingbird in management of blood sugar. I will offer evidence as to this basal/recruitable isoform relationship between GLUT1 and GLUT3.

4.1 GLUT1 and GLUT3 may provide recruitable and constitutive glucose transport in muscle, respectively.

I observed a significant increase in muscle PM GLUT1 but not PM GLUT3 associated with feeding in the hummingbird. This data matches previous studies observing the coordination of GLUT1 and 3. Stimulation of glucose transport in chicken embryo fibroblasts showed an increase of GLUT1 with hyperglycaemia, and no response of GLUT3 (Wagstaff & White, 1995). While this is surprising considering the high glucose affinity of GLUT3, the authors conclude that, such regulation occurs due to distinct regulatory requirements of mammalian and avian systems.

As in chickens, GLUT1 protein is missing in the hummingbird liver (Figure 6A). This breaks with the GLUT1/GLUT3 pairing observed in muscle cells. However, as GLUT3 shows the highest capacity for glucose transport of all other GLUT-isoforms (Byers et al., 2017), GLUT3 may facilitate the bulk of glucose transport in lieu of the missing GLUT1 protein in hummingbird hepatocytes. This is reflected in the differential handling of glucose in mammalian livers compared to muscles; a difference that largely seems to result from available and active GLUTs.

4.2 GLUT5 and GLUT2 may provide constitutive fructose transport.

While its muscle systems are usually unresponsive to fructose, the mammalian liver processes at least half of the total fructose ingested (Feinman & Fine, 2013). Fructokinase is the first enzyme encountered by imported fructose; the C-type isoform is present in mammalian hepatocytes and has a very low Michaelis-constant for fructose (K_m = 0.73mM) (Ishimoto et al., 2012). Hummingbirds have exceptional adaptations to handling fructose in their muscle. Muscle hexokinase has a phosphorylation capacity 3-fold higher for fructose than the rate of glucose-phosphorylation in mouse myocytes (Blomstrand, Challiss, Cooney, & Newsholme, 1983; Welch et al., 2018). With about half their caloric intake in the form of fructose (del Rio et al., 1992), and hummingbirds being ‘energy-maximisers’ in states of caloric excess (Diamond et al., 1986; Suarez et al., 1990), their liver must also possess a high hepatic fructose processing capacity, allowing them to utilise it in gluconeogenic, glycogenic, and importantly lipogenic pathways (Scanes, 2014).

I did not observe any change in the overall abundance of GLUT2 or GLUT5 in hepatocytes between feeding states. In the flight muscle, I did not observe any changing the PM and cytosolic abundance of GLUT2 or GLUT5. Taking into consideration the fructose-processing capacity of the avian liver (Hamer & Dickson, 1987), and a carbohydrate throughput that’s increased in periods of caloric excess, I propose that the recently fed liver is importing a significantly larger amount of fructose than rested flight muscle; largely due to the inactivity of the muscles, rather than the recruitment pattern of the fructose-specific GLUT isoforms. However, the concurrent and large (+73%) increase of GLUT3 in hummingbird hepatocyte PM (Table 4) indicates a

significant transport of glucose as well, suggesting that the fed hummingbird liver may be primed for either circulating monosaccharide.

It is important to understand how glucose (and fructose) uptake capacity responds when the flight muscles experience, not only increased sugar available via the cardiovascular system, but also increased energy demand during exercise. Therefore, it will be important to examine variation in PM GLUT abundance in fasted versus fed birds allowed to hover. Because exercise itself is known to enhance GLUT4 (and 1) translocation to the muscle cell surface in mammals, I hypothesize that GLUT1 recruitment in hover-fed bird flight muscle PM would be greater in magnitude. In contrast, I predict that GLUT3 PM expression would be similar among treatments.

5. GLUTs as regulators of hexose flux in the hummingbird model of carbohydrate management.

This study demonstrates that GLUT-isoforms in the ruby-throated hummingbird are actively regulated in a tissue-specific manner in response to being fed a sucrose meal. The work adds on to previous studies aiming to understand the physiologically extreme metabolic drive of hummingbirds and their rapid transport of exogenous fuel to intracellular combustion sites.

The findings presented here are among the first quantifying subcellular localisation of GLUT proteins in a non-chicken avian species, and the first one to compare the effects of feeding or fasting on GLUT isoforms in the PM of ruby-throated hummingbirds. Along with these findings, detailed transport kinetics are needed to improve our understanding of the specific regulation of GLUT isoforms. PM GLUT1, for example, shows complex quaternary organisation and oligomeric behaviour in relation to its function (De Zutter & Carruthers, 2013; Lloyd, Ojelabi, De Zutter, & Carruthers, 2017). GLUT1 is known to exist in ‘open’, ‘closed’, active, inactive, fused, unfused, partially fused, and endocytosable forms in the PM, usually with some protein in each state (Carruthers, 2009; Coderre, Cloherty, Zottola, & Carruthers, 1995; Hebert & Carruthers, 1992). Together, the picture of GLUT abundance, distribution and kinetics will provide the most complete understanding of the role of these crucial transporters and how they coordinate their function to meet hummingbird energy-demands and achieve metabolic homeostasis.

As most of such data is comes from studies on mammalian GLUTs, further experiments investigating substrate-specific GLUT capacities must be performed on ruby-throated hummingbirds. Experiments providing hummingbirds with glucose-only or fructose-only solutions may result in the differential recruitment of isoforms, hinting at their substrate-specificity. There is also great value in tracking labelled hexoses; allowing for the determination of deposition rates, locations, and utilisation of hexose molecules ingested. As the majority of GLUT research involves *in vitro* experiments, this understanding of *in vivo* regulation is critical Furthermore, new GLUT species are still being discovered, such as the potentially insulin-sensitive chicken GLUT12 (Coudert et al., 2015), and may add to the growing picture of hummingbird GLUT regulation.

This study also demonstrated the utility of novel, yet simple, method of protein fractionation for subsequent protein analysis for application to avian systems. The technique, slightly modified from the method of Yamamoto et al. (2016), allows for the rapid and efficient removal of PM-proteins from cytosolic ones, allowing the study of protein subcellular locations in lesser-studied, non-model organisms such as the ruby-throated hummingbird. Studying these animals is logistically complicated due to their scarcity in the wild and migratory behaviour. However, with this procedure, an abundance of data can be garnered from as little as 20mg of tissue.

In summary, while the mammalian model of hexose regulation does not fully explain the extreme transport rates observed in hummingbirds, this study provides tantalising clues to the major molecular players behind hummingbird hexose movement. I demonstrated the recruitment of glucose-specific GLUTs to the PM in response to feeding, while fructose-specific GLUTs were constitutively expressed. This work adds to the growing body of nectarivore hexose management and specifically contributes to the ever-evolving model of hummingbird hexose flux.

References

Alves, L. A., Almeida e Silva, J. B., & Giulietti, M. (2007). Solubility of D-Glucose in Water and Ethanol/Water Mixtures. *Journal of Chemical & Engineering Data*, *52*(6), 2166–2170. https://doi.org/10.1021/je700177n

Arbuckle, M. I., Kane, S., Porter, L. M., Seatter, M. J., & Gould, G. W. (1996). Structure−Function Analysis of Liver-Type (GLUT2) and Brain-Type (GLUT3) Glucose Transporters: Expression of Chimeric Transporters in *Xenopus* Oocytes Suggests an Important Role for Putative Transmembrane Helix 7 in Determining Substrate Selectivity †. *Biochemistry*, *35*(51), 16519–16527. https://doi.org/10.1021/bi962210n

Asano, T., Katagiri, H., Takata, K., Tsukuda, K., Lin, J. L., Ishihara, H., … Oka, Y. (1992). Characterization of GLUT3 protein expressed in Chinese hamster ovary cells. *Biochemical Journal*, *288*(1), 189–193. https://doi.org/10.1042/bj2880189

Barber, R. D., Harmer, D. W., Coleman, R. A., & Clark, B. J. (2005). GAPDH as a housekeeping gene: analysis of GAPDH mRNA expression in a panel of 72 human tissues. *Physiological Genomics*, *21*(3), 389–395. https://doi.org/10.1152/physiolgenomics.00025.2005

Belo, P. S., Romsos, D. R., & Leveille, G. A. (1976). Blood Metabolites and Glucose Metabolism in the Fed and Fasted Chicken. *The Journal of Nutrition*, *106*(8), 1135–1143. https://doi.org/10.1093/jn/106.8.1135

Beuchat, C. A., & Chong, C. R. (1998). Hyperglycemia in hummingbirds and its consequences for hemoglobin glycation. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *120*(3), 409–416.

Blomstrand, E., Challiss, R. A. J., Cooney, G., J., & Newsholme, E. A. (1983). Maximal activities of hexokinase, 6-phosphofructokinase, oxoglutarate dehydrogenase, and carnitine palmitoyltransferase in rat and avian muscles. *Bioscience Reports*, *3*(12).

Bode, C., Bode, J. C., Ohta, W., & Martini, G. A. (1980). Adaptive changes of the activity of enzymes involved in fructose metabolism in the liver and jejunal mucosa of rats following fructose feeding. *Research In Experimental Medicine*, *178*(1), 55–63. https://doi.org/10.1007/BF01856758

Boratyn, G. M., Schäffer, A. A., Agarwala, R., Altschul, S. F., Lipman, D. J., & Madden, T. L. (2012). Domain enhanced lookup time accelerated BLAST. *Biology Direct*, *7*(1), 12. https://doi.org/10.1186/1745-6150-7-12

Bordeaux, J., Welsh, A. W., Agarwal, S., Killiam, E., Baquero, M. T., Hanna, J. A., … Rimm, D. L. (2010). Antibody validation. *BioTechniques*, *48*(3), 197–209. https://doi.org/10.2144/000113382

Boulter, J. M., & Wang, D. N. (2001). Purification and Characterization of Human Erythrocyte Glucose Transporter in Decylmaltoside Detergent Solution. *Protein Expression and Purification*, *22*(2), 337–348. https://doi.org/10.1006/prep.2001.1440

Brady, L. J., Romsos, D. R., Brady, P. S., Bergen, W. G., & Leveille, G. A. (1978). The Effects of Fasting on Body Composition, Glucose Turnover, Enzymes and Metabolites in the Chicken. *The Journal of Nutrition*, *108*(4), 648–657. https://doi.org/10.1093/jn/108.4.648

Braun, E. J., & Sweazea, K. L. (2008). Glucose regulation in birds. *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *151*(1), 1–9. https://doi.org/10.1016/j.cbpb.2008.05.007

Burcelin, R., & Eddouks, M. (1992). Evidence that GLUT-2 mRNA and protein concentrations are decreased by hyperinsulinaemia and increased by hyperglycaemia in liver of diabetic rats, *288*, 5.

Burcelin, R., & Thorens, B. (2000). Liver Hyperplasia and Paradoxical Regulation of Glycogen Metabolism and Glucose-sensitive Gene Expression in GLUT2-null Hepatocytes: Further Evidence for the Existence of a Membrane-Based Glucose Release Pathway. *Journal of Biological Chemistry*, *275*(15), 10930–10936. https://doi.org/10.1074/jbc.275.15.10930

Byers, M., Bohannon-Stewart, A., Khwatenge, C., Alqureish, E., Alhathlol, A., Nahashon, S., & Wang, X. (2018). Absolute Quantification of Tissue Specific Expression of Glucose Transporters in Chickens, *1*, 8.

Byers, M. S., Howard, C., & Wang, X. (2017). Avian and Mammalian Facilitative Glucose Transporters. *Microarrays*, *6*(2), 7. https://doi.org/10.3390/microarrays6020007

Calder, W. A. (1994). When Do Hummingbirds Use Torpor in Nature? *Physiological Zoology*, *67*(5), 1051–1076. https://doi.org/10.1086/physzool.67.5.30163881

Carpenter, F. L., & Hixon, M. A. (1988). A New Function for Torpor: Fat Conservation in a Wild Migrant Hummingbird. *The Condor*, *90*(2), 373–378. https://doi.org/10.2307/1368565

Carpenter, F. L., Hixon, M. A., Beuchat, C. A., Russell, R. W., & Paton, D. C. (1993). Biphasic Mass Gain in Migrant Hummingbirds: Body Composition Changes, Torpor, and Ecological Significance. *Ecology*, *74*(4), 1173–1182. https://doi.org/10.2307/1940487

Carpenter, F. L., Paton, D. C., & Hixon, M. A. (1983). Weight gain and adjustment of feeding territory size in migrant hummingbirds. *Proceedings of the National Academy of Sciences*, *80*(23), 7259–7263. https://doi.org/10.1073/pnas.80.23.7259

Carruthers, A. (2009). Will the original glucose transporter isoform please stand up! *American Journal of Physiology-Endocrinology and Metabolism*, *297*(4), E836–E848. https://doi.org/10.1152/ajpendo.00496.2009

Carver, F. M., Shibley, Jr, I. A., Pennington, J. S., & Pennington, S. N. (2001). Differential expression of glucose transporters during chick embryogenesis: *Cellular and Molecular Life Sciences*, *58*(4), 645–652. https://doi.org/10.1007/PL00000887

Cavallaro, U., & Christofori, G. (2004). Cell adhesion and signalling by cadherins and Ig-CAMs in cancer. *Nature Reviews Cancer*, *4*(2), 118–132. https://doi.org/10.1038/nrc1276

Cheeseman, C., & Long, W. (2015). Structure of, and functional insight into the GLUT family of membrane transporters. *Cell Health and Cytoskeleton*, 167. https://doi.org/10.2147/CHC.S60484

Chen, C. C. W., & Welch, K. C. (2014). Hummingbirds can fuel expensive hovering flight completely with either exogenous glucose or fructose. *Functional Ecology*, *28*(3), 589–600. https://doi.org/10.1111/1365-2435.12202

Chen, K. (1945). Susceptibility of birds to insulin as compared with mammals. *Journal of Pharmacology and Experimental Therapeutics*, *84*(1), 74–77.

Clark, M. G., Rattigan, S., Barrett, E. J., & Vincent, M. A. (2008). Point:Counterpoint: There is/is not capillary recruitment in active skeletal muscle during exercise. *Journal of Applied Physiology*, *104*(3), 889–891. https://doi.org/10.1152/japplphysiol.00779.2007

Coderre, P. E., Cloherty, E. K., Zottola, R. J., & Carruthers, A. (1995). Rapid Substrate Translocation by the Multisubunit, Erythroid Glucose Transporter Requires Subunit Associations but Not Cooperative Ligand Binding. *Biochemistry*, *34*(30), 9762–9773. https://doi.org/10.1021/bi00030a014

Coudert, E., Pascal, G., Dupont, J., Simon, J., Cailleau-Audouin, E., Crochet, S., … Métayer-Coustard, S. (2015). Phylogenesis and Biological Characterization of a New Glucose Transporter in the Chicken (Gallus gallus), GLUT12. *PLOS ONE*, *10*(10), e0139517. https://doi.org/10.1371/journal.pone.0139517

De Duve, C., & Beaufay, H. (1981). A short history of tissue fractionation. *The Journal of Cell Biology*, *91*(3), 293s-299s. https://doi.org/10.1083/jcb.91.3.293s

De Zutter, J. K., & Carruthers, A. (2013). Sequence Determinants of GLUT1 Oligomerization: Analysis by Homology-Scanning Mutagenesis. *Journal of Biological Chemistry*, *288*(28), 20734–20744. https://doi.org/10.1074/jbc.M113.469023

del Rio, M., Baker, H. G., & Baker, I. (1992). Ecological and evolutionary implications of digestive processes: Bird preferences and the sugar constituents of floral nectar and fruit pulp. *Experientia*, *48*(6), 544–551. https://doi.org/10.1007/BF01920237

Di Prampero, P. E. (1985). Metabolic and circulatory limitations to VO2 max at the whole animal level. *Journal of Experimental Biology*, *115*(1), 319–331.

Diamond, J. M., Karasov, W. H., Phan, D., & Carpenter, F. L. (1986). Digestive physiology is a determinant of foraging bout frequency in hummingbirds. *Nature*, *320*(6). Retrieved from http://www.nature.com.myaccess.library.utoronto.ca/articles/320062a0.pdf

Didio, L. J. A. (1967). Myocardial ultrastructure and electrocardiograms of the hummingbird under normal and experimental conditions. *The Anatomical Record*, *159*(4), 335–351. https://doi.org/10.1002/ar.1091590402

Douard, V., & Ferraris, R. P. (2008). Regulation of the fructose transporter GLUT5 in health and disease. *American Journal of Physiology-Endocrinology and Metabolism*, *295*(2), E227–E237. https://doi.org/10.1152/ajpendo.90245.2008

Dupont, J. (2004). Early steps of insulin receptor signaling in chicken and rat: apparent refractoriness in chicken muscle. *Domestic Animal Endocrinology*, *26*(2), 127–142. https://doi.org/10.1016/j.domaniend.2003.09.004

Dupont, J. (2009). Insulin signaling in chicken liver and muscle. *General and Comparative Endocrinology*, *163*(1–2), 52–57. https://doi.org/10.1016/j.ygcen.2008.10.016

Egert, S., Nguyen, N., & Schwaiger, M. (1999). Myocardial Glucose Transporter GLUT1: Translocation Induced by Insulin and Ischemia. *Journal of Molecular and Cellular Cardiology*, *31*(7), 1337–1344. https://doi.org/10.1006/jmcc.1999.0965

Feinman, R. D., & Fine, E. J. (2013). Fructose in perspective. *Nutrition & Metabolism*, *10*(1), 45.

Feldhahn, N., Arutyunyan, A., Stoddart, S., Zhang, B., Schmidhuber, S., Yi, S.-J., … Heisterkamp, N. (2012). Environment-mediated drug resistance in Bcr/Abl-positive acute lymphoblastic leukemia. *OncoImmunology*, *1*(5), 618–629. https://doi.org/10.4161/onci.20249

Fladeby, C., Skar, R., & Serck-Hanssen, G. (2003). Distinct regulation of glucose transport and GLUT1/GLUT3 transporters by glucose deprivation and IGF-I in chromaffin cells. *Biochimica et Biophysica Acta (BBA) - Molecular Cell Research*, *1593*(2–3), 201–208. https://doi.org/10.1016/S0167-4889(02)00390-7

Fritz, J. D., Swartz, D. R., & Greaser, M. L. (1989). Factors Affecting Polyacrylamide Gel Electrophoresis and Electroblotting of High-Molecular-Weight Myofibrillar Proteins. *Analytical Biochemistry*, *180*, 205–210.

Gass, C. L., & Sutherland, G. D. (1985). Specialization by territorial hummingbirds on experimentally enriched patches of flowers: energetic profitability and learning. *Canadian Journal of Zoology*, *63*(9), 2125–2133. https://doi.org/10.1139/z85-313

Gassmann, M., Grenacher, B., Rohde, B., & Vogel, J. (2009). Quantifying Western blots: Pitfalls of densitometry. *ELECTROPHORESIS*, *30*(11), 1845–1855. https://doi.org/10.1002/elps.200800720

Griffin, H. D., Guo, K., Windsor, D., & Butterwith, S. C. (1992). Adipose Tissue Lipogenesis and Fat Deposition in Leaner Broiler Chickens. *The Journal of Nutrition*, *122*(2), 363–368. https://doi.org/10.1093/jn/122.2.363

Guma, A., Zierath, J. R., Wallberg-Henriksson, H., & Klip, A. (1995). Insulin induces translocation of GLUT-4 glucose transporters in human skeletal muscle. *American Journal of Physiology-Endocrinology and Metabolism*, *268*(4), E613–E622. https://doi.org/10.1152/ajpendo.1995.268.4.E613

Hainsworth, F. R., & Wolf, L. L. (1972). Crop volume, nectar concentration and hummingbird energetics. *Comparative Biochemistry and Physiology Part A: Physiology*, *42*(2), 359–366. https://doi.org/10.1016/0300-9629(72)90117-X

Hamer, M. J., & Dickson, A. J. (1987). Developmental changes in hepatic fructose 2,6-bisphosphate content and phosphofructokinase-1 activity in the transition of chicks from embryonic to neonatal nutritional environment. *Biochemical Journal*, *245*(1), 35–39. https://doi.org/10.1042/bj2450035

Hargrove, J. L. (2005). Adipose energy stores, physical work, and the metabolic syndrome: lessons from hummingbirds. *Nutrition Journal*, *4*(1). https://doi.org/10.1186/1475-2891-4-36

Hazelwood, R. L. (1973). The Avian Endocrine Pancreas. *American Zoologist*, *13*(3), 699–709. https://doi.org/10.1093/icb/13.3.699

Hebert, D. N., & Carruthers, A. (1992). Glucose transporter oligomeric structure determines transporter function. Reversible redox-dependent interconversions of tetrameric and dimeric GLUT1. *Journal of Biological Chemistry*, *267*(33), 23829–23838.

Helliwell, P. A., Richardson, M., Affleck, J., & Kellett, G. L. (2000). Regulation of GLUT5, GLUT2 and intestinal brush-border fructose absorption by the extracellular signal-regulated kinase, p38 mitogen-activated kinase and phosphatidylinositol 3-kinase intracellular signalling pathways: implications for adaptation to diabetes. *Biochemical Journal*, *350*(Pt 1), 163–169.

Hou, L., Verdirame, M., & Welch, K. C. (2015). Automated tracking of wild hummingbird mass and energetics over multiple time scales using radio frequency identification (RFID) technology. *Journal of Avian Biology*, *46*(1), 1–8. https://doi.org/10.1111/jav.00478

Hou, L., & Welch, K. C. (2016). Premigratory ruby-throated hummingbirds, Archilochus colubris , exhibit multiple strategies for fuelling migration. *Animal Behaviour*, *121*, 87–99. https://doi.org/10.1016/j.anbehav.2016.08.019

Ishimoto, T., Lanaspa, M. A., Le, M. T., Garcia, G. E., Diggle, C. P., MacLean, P. S., & Rivard, C. J. (2012). Opposing effects of fructokinase C and A isoforms on fructose-induced metabolic syndrome in mice. *Proceedings of the National Academy of Sciences*.

Johansen, K. (1987). Respiratory Properties of Blood and Myoglobin in Hummingbirds. *Society for Integrative and Comparative Biology*, *60*(2), 269–278.

Jorgensen, P. L., Håkansson, K. O., & Karlish, S. J. D. (2003). Structure and Mechanism of Na,K-ATPase: Functional Sites and Their Interactions. *Annual Review of Physiology*, *65*(1), 817–849. https://doi.org/10.1146/annurev.physiol.65.092101.142558

Kaiser, N., Leibowitz, G., & Nesher, R. (2003). Glucotoxicity and ß-Cell Failure in Type 2 Diabetes Mellitus. *JOURNAL OF PEDIATRIC ENDOCRINOLOGY*, 18.

Karasov, W. H. (2017). Integrative physiology of transcellular and paracellular intestinal absorption. *The Journal of Experimental Biology*, *220*(14), 2495–2501. https://doi.org/10.1242/jeb.144048

Karim, S. (2012). Hepatic expression and cellular distribution of the glucose transporter family. *World Journal of Gastroenterology*, *18*(46), 6771. https://doi.org/10.3748/wjg.v18.i46.6771

Kayano, T., & Bell, G. I. (1988). Evidence for a family of human glucose transporter-like proteins. Sequence and gene localization of a protein expressed in fetal skeletal muscle and other tissues. *Journal of Biological Chemistry*, *263*(30), 15248–15248.

Kayano, T., & Bell, G. I. (1990). Human facilitative glucose transporters. Isolation, functional characterization, and gene localization of cDNAs encoding an isoform (GLUT5) expressed in small intestine, kidney, muscle, and adipose tissue and an unusual glucose transporter pseudogene-like sequence (GLUT6). *Journal of Biological Chemistry*, *265*(22), 13276–13282.

Kellett, G. L., Brot-Laroche, E., Mace, O. J., & Leturque, A. (2008). Sugar Absorption in the Intestine: The Role of GLUT2. *Annual Review of Nutrition*, *28*(1), 35–54. https://doi.org/10.1146/annurev.nutr.28.061807.155518

Klip, A., & Vranic, M. (2006). Muscle, liver, and pancreas: Three Musketeers fighting to control glycemia. *American Journal of Physiology-Endocrinology and Metabolism*, *291*(6), E1141–E1143. https://doi.org/10.1152/classicessays.00043.2006

Kono, T., Nishida, M., Nishiki, Y., Seki, Y., Sato, K., & Dr, Y. A. (2005). Characterisation of glucose transporter (GLUT) gene expression in broiler chickens. *British Poultry Science*, *46*(4), 510–515. https://doi.org/10.1080/00071660500181289

Kumagai, A. K., Dwyer, K. J., & Pardridge, W. M. (1994). Differential glycosylation of the GLUT1 glucose transporter in brain capillaries and choroid plexus. *Biochimica et Biophysica Acta (BBA) - Biomembranes*, *1193*(1), 24–30. https://doi.org/10.1016/0005-2736(94)90328-X

Lasiewski, R. C. (1963). Oxygen Consumption of Torpid, Resting, Active, and Flying Hummingbirds. *Physiological Zoology*, *36*(2), 122–140. https://doi.org/10.1086/physzool.36.2.30155436

Lasiewski, R. C. (1964). Body temperatures, heart and breathing rate, and evaporative water loss in hummingbirds. *Physiological Zoology*, *37*(2), 212–223.

Li, N., Luo, H., & Liu, S. (2004). A new method for the determination of the critical micelle concentration of Triton X-100 in the absence and presence of β-cyclodextrin by resonance Rayleigh scattering technology. *Spectrochimica Acta Part A: Molecular and Biomolecular Spectroscopy*, *60*(8–9), 1811–1815. https://doi.org/10.1016/j.saa.2003.09.018

Lloyd, K. P., Ojelabi, O. A., De Zutter, J. K., & Carruthers, A. (2017). Reconciling contradictory findings: Glucose transporter 1 (GLUT1) functions as an oligomer of allosteric, alternating access transporters. *Journal of Biological Chemistry*, *292*(51), 21035–21046. https://doi.org/10.1074/jbc.M117.815589

Maher, F., & Vannucci, S. J. (1993). Glucose Transporter Isoforms in Brain: Absence of GLUT3 from the Blood—Brain Barrier. *Journal of Cerebral Blood Flow & Metabolism*, *13*(2), 342–345. https://doi.org/10.1038/jcbfm.1993.43

Masterson, J., & O'Dea, S. (2007). Posttranslational Truncation of E-Cadherin and Significance for Tumour Progression. *Cells Tissues Organs*, *185*(1–3), 175–179. https://doi.org/10.1159/000101318

Mathieu-Costello, O., Suarez, R., & Hochachka, P. W. (1992). Capillary-to-fiber geometry and mitochondrial density in hummingbird flight muscle. *Respiration Physiology*, *89*(1), 113–132.

Mathoo, J. M., Shi, Z. Q., Klip, A., & Vranic, M. (1999). Opposite effects of acute hypoglycemia and acute hyperglycemia on glucose transport and glucose transporters in perfused rat skeletal muscle. *Diabetes*, *48*(6), 1281–1288. https://doi.org/10.2337/diabetes.48.6.1281

McCulloch, L. J., van de Bunt, M., Braun, M., Frayn, K. N., Clark, A., & Gloyn, A. L. (2011). GLUT2 (SLC2A2) is not the principal glucose transporter in human pancreatic beta cells: Implications for understanding genetic association signals at this locus. *Molecular Genetics and Metabolism*, *104*(4), 648–653. https://doi.org/10.1016/j.ymgme.2011.08.026

McMillin, S. L., Schmidt, D. L., Kahn, B. B., & Witczak, C. A. (2017). Glucose Transporter 4 (GLUT4) is Not Necessary for Overload-Induced Glucose Uptake or Hypertrophic Growth in Mouse Skeletal Muscle, 48.

McWhorter, T. J. (2006). Hummingbirds rely on both paracellular and carrier-mediated intestinal glucose absorption to fuel high metabolism. *Biology Letters*, *2*(1), 131–134. https://doi.org/10.1098/rsbl.2005.0388

McWilliams, S. R., Guglielmo, C., Pierce, B., & Klaassen, M. (2004). Flying, fasting, and feeding in birds during migration: a nutritional and physiological ecology perspective. *Journal of Avian Biology*, *35*(5), 377–393. https://doi.org/10.1111/j.0908-8857.2004.03378.x

Mitsumoto, Y., & Klip, A. (1992). Developmental Regulationof the Subcellular Distribution and Glycosylation of GLUT1 and GLUT4 Glucose Transporters during Myogenesis of L6 Muscle Cells. *The Journal of Biological Chemistry*, *267*(7), 4957–4962.

Myrka, A. M., & Welch, K. C. (2018). Evidence of high transport and phosphorylation capacity for both glucose and fructose in the ruby-throated hummingbird (Archilochus colubris). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *224*, 253–261. https://doi.org/10.1016/j.cbpb.2017.10.003

Neville, D. M. (1960). The Isolation Of A Cell Membrane Fraction From Rat Liver. *The Journal of Cell Biology*, *8*(2), 413–422. https://doi.org/10.1083/jcb.8.2.413

Nicolson, S. W., & Fleming, P. A. (2003). Nectar as food for birds: the physiological consequences of drinking dilute sugar solutions. *Plant Systematics and Evolution*, *238*(1–4), 139–153. https://doi.org/10.1007/s00606-003-0276-7

Palade, G. (1975). Intracellular Aspects of the Process of Protein Synthesis. *Science*, *189*(4200), 347–358.

Pencek, R. R., James, F., Lacy, D. B., Jabbour, K., Williams, P. E., Fueger, P. T., & Wasserman, D. H. (2003). Interaction of Insulin and Prior Exercise in Control of Hepatic Metabolism of a Glucose Load. *Diabetes*, *52*(8), 1897–1903. https://doi.org/10.2337/diabetes.52.8.1897

Pollock, C. (2002). Carbohydrate regulation in avian species. *Journal of Exotic Pet Medicine*, *11*(2), 57–64.

Powers, D. R., & Nagy, K. A. (1988). Field Metabolic Rate and Food Consumption by Free-Living Anna's Hummingbirds (Calypte anna). *Physiological Zoology*, *61*(6), 500–506. https://doi.org/10.1086/physzool.61.6.30156158

Price, E. R., Brun, A., Caviedes-Vidal, E., & Karasov, W. H. (2015). Digestive Adaptations of Aerial Lifestyles. *Physiology*, *30*(1), 69–78. https://doi.org/10.1152/physiol.00020.2014

Ramenofsky, M., Savard, R., & Greenwood, M. R. C. (1999). Seasonal and diel transitions in physiology and behavior in the migratory dark-eyed junco. *Comparative Biochemistry and Physiology Part A: Molecular & Integrative Physiology*, *122*(4), 385–397. https://doi.org/10.1016/S1095-6433(99)00013-6

Roberts, T. J., Weber, J.-M., Hoppeler, H., Weibel, E. R., & Taylor, C. R. (1996). Design Of The Oxygen And Substrate Pathways: II. Defining The Upper Limits Of Carbohydrate And Fat Oxidation, 8.

Samih, N., Hovsepian, S., Notel, F., Prorok, M., Zattara-Cannoni, H., Mathieu, S., … El-Battari, A. (2003). The impact of N- and O-glycosylation on the functions of Glut-1 transporter in human thyroid anaplastic cells. *Biochimica et Biophysica Acta (BBA) - General Subjects*, *1621*(1), 92–101. https://doi.org/10.1016/S0304-4165(03)00050-3

Scanes, C. G. (Ed.). (2014). *Sturkie's Avian Physiology* (6th Edition). Elsevier.

Scheepers, A., Joost, H., & Schurmann, A. (2004). The glucose transporter families SGLT and GLUT: molecular basis of normal and aberrant function. *Journal of Parenteral and Enteral Nutrition*, *28*(5), 364–371. https://doi.org/10.1177/0148607104028005364

Seki, Y. (2003). Broiler chickens (Ross strain) lack insulin-responsive glucose transporter GLUT4 and have GLUT8 cDNA. *General and Comparative Endocrinology*, *133*(1), 80–87. https://doi.org/10.1016/S0016-6480(03)00145-X

Solomon, T. P. J., Knudsen, S. H., Karstoft, K., Winding, K., Holst, J. J., & Pedersen, B. K. (2012). Examining the Effects of Hyperglycemia on Pancreatic Endocrine Function in Humans: Evidence for *in Vivo* Glucotoxicity. *The Journal of Clinical Endocrinology & Metabolism*, *97*(12), 4682–4691. https://doi.org/10.1210/jc.2012-2097

Sové, R. J., Goldman, D., & Fraser, G. M. (2017). A computational model of the effect of capillary density variability on oxygen transport, glucose uptake, and insulin sensitivity in prediabetes. *Microcirculation*, *24*(2), e12342. https://doi.org/10.1111/micc.12342

Suarez, R. (1992). Hummingbird flight: Sustaining the highest mass-specific metabolic rates among vertebrates. *Experientia*, *48*(6), 565–570. https://doi.org/10.1007/BF01920240

Suarez, R. (1998). Oxygen and the Upper Limits to Animal Design and Performance. *The Journal of Experimental Biology*, *201*, 1065–1072.

Suarez, R., Brown, G. S., & Hochachka, P. W. (1986). Metabolic sources of energy for hummingbird flight. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *251*(3), R537–R542. https://doi.org/10.1152/ajpregu.1986.251.3.R537

Suarez, R., Brownsey, R. W., Vogl, W., Brown, G. S., & Hochachka, P. W. (1988). Biosynthetic capacity of hummingbird liver. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *255*(5), R699–R702. https://doi.org/10.1152/ajpregu.1988.255.5.R699

Suarez, R., Lighton, J. R., Brown, G. S., & Mathieu-Costello, O. (1991). Mitochondrial respiration in hummingbird flight muscles. *Proceedings of the National Academy of Sciences*, *88*(11), 4870–4873.

Suarez, R., Lighton, J. R., Moyes, C. D., Brown, G. S., Gass, C. L., & Hochachka, P. W. (1990). Fuel selection in rufous hummingbirds: ecological implications of metabolic biochemistry. *Proceedings of the National Academy of Sciences*, *87*(23), 9207–9210. https://doi.org/10.1073/pnas.87.23.9207

Suarez, R., & Welch, K. (2017). Sugar Metabolism in Hummingbirds and Nectar Bats. *Nutrients*, *9*(7), 743. https://doi.org/10.3390/nu9070743

Suarez, R., & Welch, K. C. (2011). The sugar oxidation cascade: aerial refueling in hummingbirds and nectar bats. *Journal of Experimental Biology*, *214*(2), 172–178. https://doi.org/10.1242/jeb.047936

Sweazea, K. L., & Braun, E. J. (2006). Glucose transporter expression in English sparrows (Passer domesticus). *Comparative Biochemistry and Physiology Part B: Biochemistry and Molecular Biology*, *144*(3), 263–270. https://doi.org/10.1016/j.cbpb.2005.12.027

Sweazea, K. L., Braun, E. J., & Sparr, R. (2017). Novel role of insulin in the regulation of glucose excretion by mourning doves (Zenaida macroura). *Zoology*, *122*, 58–62. https://doi.org/10.1016/j.zool.2017.02.006

Thoidis, G., Kupriyanova, T., Cunningham, J. M., Chen, P., Cadel, S., Foulon, T., … Kandror, K. V. (1999). Glucose Transporter Glut3 Is Targeted to Secretory Vesicles in Neurons and PC12 Cells. *Journal of Biological Chemistry*, *274*(20), 14062–14066. https://doi.org/10.1074/jbc.274.20.14062

Thorens. (1996). Glucose transporters in the regulation of intestinal, renal, and liver glucose fluxes. *American Journal of Physiology-Gastrointestinal and Liver Physiology*, *270*(4), G541–G553. https://doi.org/10.1152/ajpgi.1996.270.4.G541

Thorens, B., & Cheng, Z.-Q. (1990). Liver glucose transporter: a basolateral protein in hepatocytes and intestine and kidney cells. *American Journal of Physiology-Cell Physiology*, *259*(6), 1–1.

Thorens, & Mueckler, M. (2010). Glucose transporters in the 21st Century. *American Journal of Physiology-Endocrinology and Metabolism*, *298*(2), E141–E145. https://doi.org/10.1152/ajpendo.00712.2009

Uldry, M., & Thorens, B. (2004). The SLC2 family of facilitated hexose and polyol transporters. *European Journal of Physiology*, *447*(5), 480–489. https://doi.org/10.1007/s00424-003-1085-0

Vannucci, S. J. (2008). Developmental Expression of GLUT1 and GLUT3 Glucose Transporters in Rat Brain. *Journal of Neurochemistry*, *62*(1), 240–246. https://doi.org/10.1046/j.1471-4159.1994.62010240.x

Velten, B. P., & Welch, K. C. (2014). Myosin heavy-chain isoforms in the flight and leg muscles of hummingbirds and zebra finches. *American Journal of Physiology-Regulatory, Integrative and Comparative Physiology*, *306*(11), R845–R851. https://doi.org/10.1152/ajpregu.00041.2014

Verdon, G., Nomura, N., Joo Kang, H., Shimamura, T., Nomura, Y., Abdul Hussien, S., … Drew, D. (2016). Structure and Mechanism of the Mammalian Fructose Transporter GLUT5. *Biophysical Journal*, *110*(3), 137a. https://doi.org/10.1016/j.bpj.2015.11.783

Wagstaff, P., & White, M. K. (1995). Characterization of the Avian GLUTI Glucose Transporter: Differential Regulation of GLUT1 and GLUT3 in Chicken Embryo Fibroblasts. *Molecular Biology of the Cell*, *6*, 15.

Wasserman, D. H. (2009). Four grams of glucose. *American Journal of Physiology-Endocrinology and Metabolism*, *296*(1), E11–E21. https://doi.org/10.1152/ajpendo.90563.2008

Wasserman, D. H., Kang, L., Ayala, J. E., Fueger, P. T., & Lee-Young, R. S. (2011). The physiological regulation of glucose flux into muscle in vivo. *Journal of Experimental Biology*, *214*(2), 254–262. https://doi.org/10.1242/jeb.048041

Wasserman, D. H., Spalding, J. A., Lacy, D. B., Colburn, C. A., Goldstein, R. E., & Cherrington, A. D. (1989). Glucagon is a primary controller of hepatic glycogenolysis and gluconeogenesis during muscular work. *American Journal of Physiology-Endocrinology and Metabolism*, *257*(1), E108–E117. https://doi.org/10.1152/ajpendo.1989.257.1.E108

Weber, J.-M. (2011). Metabolic fuels: regulating fluxes to select mix. *Journal of Experimental Biology*, *214*(2), 286–294. https://doi.org/10.1242/jeb.047050

Weber, J.-M., Roberts, T. J., Vock, R., Weibel, E. R., & Taylor, C. R. (1996). Design Of The Oxygen And Substrate Pathways: III. Partitioning Energy Provision From Carbohydrates, 8.

Welch, K., Bakken, B., del Rio, M., & Suarez, R. (2006). Hummingbirds Fuel Hovering Flight with Newly Ingested Sugar. *Physiological and Biochemical Zoology*, *79*(6), 1082–1087.

Welch, K. C., Allalou, A., Sehgal, P., Cheng, J., & Ashok, A. (2013). Glucose Transporter Expression in an Avian Nectarivore: The Ruby-Throated Hummingbird (Archilochus colubris). *PLoS ONE*, *8*(10), e77003. https://doi.org/10.1371/journal.pone.0077003

Welch, K. C., Altshuler, D. L., & Suarez, R. (2007). Oxygen consumption rates in hovering hummingbirds reflect substrate-dependent differences in P/O ratios: carbohydrate as a `premium fuel'. *Journal of Experimental Biology*, *210*(12), 2146–2153. https://doi.org/10.1242/jeb.005389

Welch, K. C., Myrka, A. M., Ali, R. S., & Dick, M. F. (2018). The Metabolic Flexibility of Hovering Vertebrate Nectarivores. *Physiology*, *33*(2), 127–137. https://doi.org/10.1152/physiol.00001.2018

Welch, K. C., & Suarez, R. (2007). Oxidation rate and turnover of ingested sugar in hovering Anna's (Calypte anna) and rufous (Selasphorus rufus) hummingbirds. *Journal of Experimental Biology*, *210*(12), 2154–2162. https://doi.org/10.1242/jeb.005363

Wood, I. S., & Trayhurn, P. (2003). Glucose transporters (GLUT and SGLT): expanded families of sugar transport proteins. *British Journal of Nutrition*, *89*(01), 3. https://doi.org/10.1079/BJN2002763

Workman, R. E., Myrka, A. M., Wong, G. W., Tseng, E., Welch, K. C., & Timp, W. (2018). Single-molecule, full-length transcript sequencing provides insight into the extreme metabolism of the ruby-throated hummingbird Archilochus colubris. *GigaScience*, *7*(3). https://doi.org/10.1093/gigascience/giy009

Yamamoto, N., Yamashita, Y., Yoshioka, Y., Nishiumi, S., & Ashida, H. (2016). Rapid Preparation of a Plasma Membrane Fraction: Western Blot Detection of Translocated Glucose Transporter 4 from Plasma Membrane of Muscle and Adipose Cells and Tissues. *Curr Protoc Protein Sci.*, *85:29*(18), 1–29.

Yang, J., & Holman, G., D. (1993). Comparison of GLUT4 and GLUT 1 Subcellular Trafficking in Basal and Insulin-stimulated 3T3-Ll Cells. *The Journal of Biological Chemistry*, *268*(7), 4600–4603.

Zhang, G., Li, C., Gilbert, M. T. P., Jarvis, E. D., Wang, J., & The Avian Genome Consortium. (2014). Comparative genomic data of the Avian Phylogenomics Project. *GigaScience*, *3*(1). https://doi.org/10.1186/2047-217X-3-26

Zhang, W., Sumners, L. H., Siegel, P. B., Cline, M. A., & Gilbert, E. R. (2013). Quantity of glucose transporter and appetite-associated factor mRNA in various tissues after insulin injection in chickens selected for low or high body weight. *Physiological Genomics*, *45*(22), 1084–1094. https://doi.org/10.1152/physiolgenomics.00102.2013

Zhao, F.-Q., & Keating, A. F. (2007). Functional Properties and Genomics of Glucose Transporters. *Current Genomics*, *8*(2), 113–128.

Zimmet, P. Z., Magliano, D. J., Herman, W. H., & Shaw, J. E. (2014). Diabetes: a 21st century challenge. *The Lancet Diabetes & Endocrinology*, *2*(1), 56–64. https://doi.org/10.1016/S2213-8587(13)70112-8

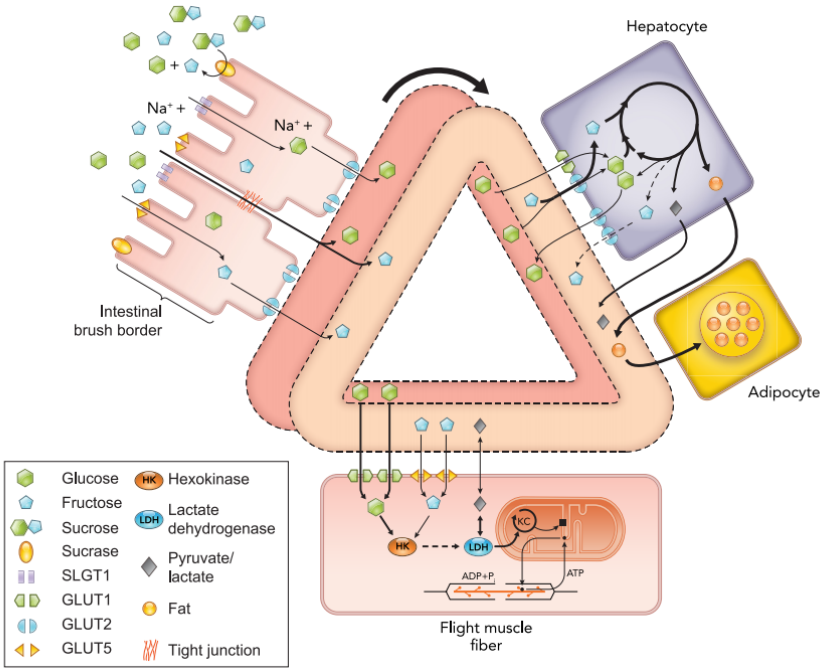
Appendices

Appendix 1: Description of known mammalian GLUTs, their localisation and substrate affinty.

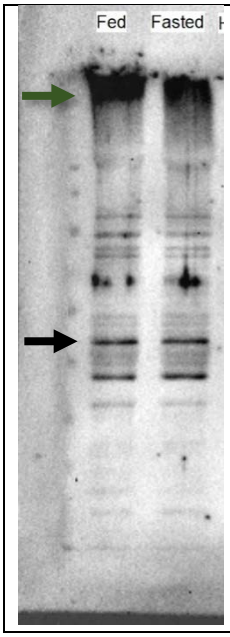
Glucose Transporter	Localisation	Function	Glucose Affinity	Other Substrates
1	Throughout body, blood-tissue barriers	Catalyses rate-limiting step in glucose transport	$K_m \approx$ 3mM	Galactose, mannose, glucosamine
(Thorens & Mueckler 2009; Carruthers 2009; Lemieux 2007; Mueckler 1985)				
2	Intestine, kidney	Catalyses first step in glucose-stimulated insulin secretion	$K_m \approx$ 17mM	Fructose, glucosamine, galactose, mannose
(Thorens 1990; Wright & Turk 2003; Uldry & Bernard 2004)				
3	Neurons lymphocytes, macrophages, platelets, monocytes, testes, skeletal muscle	Neuronal tissue glucose transport	$K_m \approx$1.4mM	Galactose, mannose, maltose, xylose, dehydroascorbic acid
(Simpson 2008; Haber 1993; Stuart 1999)				
4	Adipocytes, skeletal muscle	Insulin-regulated whole-body homeostasis	$K_m \approx$ 5mM	Dehydroascorbic acid, glucosamine
(James 1988; Larance 2008; Kasahara 1997)				
5	Intestine, brain, adipocytes, testes, skeletal muscle	Fructose transport	N/A	Fructose affinity: $K_m \approx$ 6mM
(Douard & Ferraris 2008; Rand 1993; Burant 1992)				

Appendix 2: Comparison of known avian GLUT isoforms and their homology to humans. Data was aggregated from (Byers et al., 2017; Myrka & Welch, 2018; Sweazea & Braun, 2006; Welch et al., 2013) and homology to humans was calculated using NCBI BLAST (Boratyn et al., 2012).

GLUT	Localisation	Feature	Chicken to human sequence homology	Hummingbird to human sequence homology	Substrates
1	Ubiquitous	Basal glucose transport	80%	88%	Galactose, mannose, glucosamine
2	Liver, Pancreas, Intestine, Kidney	insulin dependent [33].	65%	64%	Fructose, Glucose, Galactose
3	Neurons, Liver, skeletal muscle	Insulin dependent	70%	73%	Glucose
4	Not found	Absence	N/A	N/A	N/A
5	Intestine, brain, adipocytes, testes, skeletal muscle	Fructose transport	N/A	68%	Fructose



Appendix 3: Major sites of transmembrane glucose (hexagons) and fructose (pentagons) facilitated and passive transport in the hummingbirds. The tissue-specific locations are represented for major GLUT and SGLT isoforms as well key enzymes involved in glycolysis. Hexose movement from the gut (top-left) to hepatocytes (top-right) and flight muscles (bottom) are depicted. Reprinted with permission from (Welch et al., 2018).



Appendix 4: Representative Western blot of high molecular weight staining observed with the initial homogenisation method as described in Chapter 2 section 1.3. “Fed” represents flight muscle of fed hummingbirds while “Fasted’ represents fasted individuals. The black arrow indicates the known size of GLUT1 (~47kDa), while the green arrow indicates the larger, unwanted high-molecular weight (>200kDa) non-specific staining.