Tracing Nutrient Sources to Lipid Production in Birds and Insects Using Stable Isotope (δ13C, δ2H) Tracers: Implications for Nutritional Physiology of Migratory Species

Libesha Anparasan, The University of Western Ontario

Supervisor: Hobson, Keith A., The University of Western Ontario
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Abstract

Using stable isotope measurements of inert tissues to determine origins and migratory patterns is well established. However, isotopically determining nutritional origins of lipids, the primary fuel of migration, has not been attempted. I explored isotopic links between diet and stored lipids in captive White-throated Sparrows (*Zonotrichia albicollis*) and true armyworm moths (*Mythimna unipuncta*) using δ¹³C and δ²H measurements. Isotopic discrimination between body lipids and diet was established as linear calibration functions. Isotopic uptake following a diet switch in moths was used to trace lipid accumulation over time. Isotopic correlations between breath metabolic by-products of fed and fasted sparrows were explored as indicators of lipid use. This study established isotopic (δ¹³C, δ²H) linkages between diet and stored lipids for migratory insects and passerines and advocates the use of stable isotopes in lipids as a tool to evaluate nutrient origins and allocation strategies in a variety of migratory species.

Keywords

lipids, isotopic tracing, migration, deuterium, carbon-13, White-throated Sparrow, true armyworm moth
Summary for Lay Audience

Migration is a phenomenon that occurs across a large variety of species and is key to the survival of many organisms. The primary fuel for migration in most organisms is lipids but determining where these lipids are synthesized, how they are used, and how they are replenished is difficult using traditional means. Tracking migration has benefitted from using intrinsic markers such as naturally occurring stable isotopes. Isotopes are forms of the same element with different kinetic properties due to differences in atomic mass. These isotopes differ across the natural environment due to a variety of biogeochemical processes and are integrated into animal tissues, such as feathers or claws, often in fixed and traceable patterns. Though lipids contain primarily carbon and hydrogen, the traceability of lipids through isotopes of these elements for the purposes of tracking nutritional strategies in migratory organisms has not been explored. My study investigated the isotopic relationship between diet and body lipids using stable hydrogen and carbon isotopes in two model organisms, the White-throated Sparrow and the true armyworm moth. By providing the study organisms with diets that were grouped via a 2x2 experimental design with combinations of high $^{13}$C, low $^{13}$C, high $^2$H, and low $^2$H, I investigated if different diets would lead to differential isotopic values in lipids synthesized from the diet. Carbon and hydrogen isotopic values of the lipids corresponded strongly with the diet from which they were made. Additionally, isotopic dietary changes performed on the moths were reflected in the uptake and storage of lipids over time. When sparrows were fasted and induced to burn lipids, the breath CO$_2$ and water vapour arising from lipid metabolism also reflected isotopic distinctions of the diet treatments. In conclusion, isotopic connections between diet and stored lipids for migratory insects and sparrows were established. This study advocates the use isotopic measurements of lipids as a tool to evaluate nutrient origins and allocation strategies in a variety of migratory species.
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List of Abbreviations

QMR = quantitative magnetic resonance
l = litre
min = minute
°C = degrees Celsius
‰ = parts per thousand
g = gram
Δ = arithmetic discrimination
C = carbon
H = hydrogen
O = oxygen
δ²H = deuterium isotopic ratio value
δ¹³C = carbon isotopic ratio value
δ¹⁵N = nitrogen isotopic ratio value
δ³⁴S = sulphur isotopic ratio value
¹³C = carbon-13
²H = deuterium
kPa = kilo Pascals
RH = relative humidity
U = unspiked tap water
S = deuterium spiked water
Δ¹³C = arithmetic discrimination of δ¹³C
Δ²H = arithmetic discrimination of δ²H
IAEA = International Atomic Energy Agency
GNIP = Global Network of Isotopes in Precipitation
USGS = United States Geological Survey
VSMOW2 = Vienna Standard Mean Ocean Water 2
CBS = Caribou Hoof Standard
KHS = Kudu Horn Standard
OA-ICOS = Off-Axis Integrated Cavity Output Spectroscopy
SLAP = Standard Light Antarctic Precipitation
SD = standard deviation
VPDB = Vienna Pee Dee Belemnite
ANOVA = analysis of variance
GLM = generalized linear model
\( \delta^2H_{\text{lip}} \) = deuterium isotopic value in body lipid
\( \delta^2H_c \) = deuterium isotopic value in dietary carbohydrates
\( \delta^{13}C_{\text{lip}} \) = carbon isotopic value in body lipid
\( \delta^{13}C_c \) = carbon isotopic value in dietary carbohydrate
\( \delta^2H_{\text{wing}} \) = deuterium isotopic value in wing
\( \delta^2H_{\text{precip}} \) = deuterium isotopic value in environmental precipitation
CAM = Crassulacean acid metabolism
RuBP = Rubisco
CF-IRMS = Continuous-flow isotope ratio mass spectrometry
LSIS-AFAR = Laboratory for Stable Isotope Science – Advanced Facility for Avian Research
Chapter 1

1 General Introduction

1.1 Tracking migration

Migration is defined as the seasonal undistracted, persistent, and straight-pathed locomotor activity outside of an individual organism’s home range (Dingle, 2014). This key component of the life history of many organisms occurs in response to numerous factors but is primarily linked to seasonal changes in the availability of resources and occurs at various spatial scales (Hobson et al., 2019b; McWilliams et al., 2001, 2004). Understanding the migratory movements of organisms can provide us with a wealth of information about an individual’s life history, physiology, and behavior, which can in turn be used to aid in their conservation and management (Lennox et al., 2016; Meretsky et al., 2011). Current changes in environmental cues due to climate change and anthropogenic land-use practices have caused alterations to migration patterns of several species (Robinson et al., 2009). Many migratory species rely on multiple fuelling sites to accumulate lipids to successfully migrate to desired locations (McWilliams et al. 2004; Robinson et al., 2009). Inability of migrants to build lipids due to a paucity of good refuelling sites decreases the chances of successful migration. For example, a study conducted on populations of Red Knots (Calidris canutus rufa) in Delaware Bay, USA, found that populations were declining primarily due to the effects of late arrival times and reduced refuelling patterns caused by loss of refuelling sites (Baker et al., 2004).

Tracking migration and the changes in migration patterns of animals is essential to understanding conservation and management needs of migratory organisms as they change overtime with the shifting environments (Fraser et al., 2018).

Earlier tracking methods used to study animal migration were primarily extrinsic (externally applied) tags and the use of mark-recapture analyses (Hobson et al., 2019b). Although these methods can be efficient and are commonly used, they suffer from poor or biased recapture probability, common bias to origin of where animals are marked, and possible effects on organism behavior (Barron et al., 2010; O’Brien, 2015). Intrinsic
markers are not biased to origin of capture or release, only require one capture, and enable researchers to gain information on thousands of individuals, often inexpensively and without influencing behavior of the organism (i.e. the animal was not required to carry any instrumentation prior to capture (Hobson et al., 2019b)). Though intrinsic markers help to mitigate some of the concerns presented by extrinsic markers, they are not without shortcomings. The resolution of origins using intrinsic markers is not as high as with geolocating tags or other extrinsic means. Intrinsic markers such as stable isotopes can constrain the location of origin to a specific region but cannot pinpoint the exact location without the use of multiple markers (Bowen & West, 2019). Despite this, intrinsic markers such as stable isotopes have been used widely to provide us with large-scale patterns of origins and migratory movement across a variety of organisms (Hobson & Wassenaar, 2019).

1.2 Using stable isotopes in migratory studies

Tracking of migratory organisms is an area of study that is constantly evolving, and a powerful established intrinsic tool is the measurement in animal tissues of naturally occurring stable isotopes of the light elements (C, H, N, O, S) found in food webs (Hobson et al., 2010; Wassenaar, 2019). Stable isotopes are forms of the same element differing only in atomic mass (i.e. the number neutrons). Stable isotopes of any element are chemically the same but differ in their rates of reaction (i.e. kinetic properties) in various biogeochemical processes (Hobson, 1999; Peterson & Fry, 1987). Typically, heavier isotopes form stronger bonds and occur in lower energy states (Hobson et al., 2010). The heavy isotopes of an element (e.g. $^{13}$C vs. $^{12}$C, $^2$H vs. $^1$H) are rare and it is the ratio of the rare heavy to more common light (e.g. $^{13}$C/$^{12}$C, $^2$H/$^1$H) isotopes in nature that provides important information (Hobson, 1999; Wassenaar, 2019). Through the process of isotopic discrimination due to various physiological and biogeochemical processes, isotopic ratios of heavy to light isotopes differ across a region, often in understood or described patterns (Hobson, 1999; Wassenaar, 2019). Stable isotope values are expressed in “δ” notation, describing the parts per mille (thousand) difference in ratios of the heavier to lighter isotope of an element in a sample and a reference material:

$$\delta X = (R_{\text{sample}}/R_{\text{standard}} - 1) \times 1000 \%_\circ$$
where X is the heavy isotope (e.g. $^2$H) and R is the ratio of heavy to lighter isotope (e.g. $^2$H/$^1$H) (Peterson & Fry, 1987; Hobson, 1999; Werner & Brand, 2001).

All stable isotope δ values are a measure, then, of how much the stable isotope ratio of a sample differs from that of a standard. Internationally accepted standard reference materials are arbitrary but have been chosen and administered primarily by the International Atomic Energy Agency (IAEA) in Vienna (Gröning, 2004; Wassenaar, 2019). This is done so that all reported isotopic values are comparable. This study focuses on the stable isotopes of hydrogen and carbon, whose international reference standards are Vienna Standard Mean Ocean Water (VSMOW) and Vienna Pee Dee Belemnite (VPDB), respectively (Gröning et al., 1999; Wassenaar, 2019).

Spatial isotopic patterns in environmental substrates or food webs have been termed “isoscapes” (West et al., 2008) and have been used to trace movements of migratory animals in both terrestrial and marine biomes (Bowen & West, 2019). The use of isoscapes for tracking is based on the fact that organisms incorporate nutrients from the environment into tissues through various metabolic processes and the isotopic values in diets can be transferred predictably to those in animal tissues (i.e. they remain traceable) (DeNiro & Epstein, 1977; Hobson & Wassenaar, 2019).

Changes between substrate and tissue isotope values are usually expressed as a fixed arithmetic factor (Δ) (Lajtha & Michener, 1994; Hobson 1999) or, more appropriately, as a transformation function or calibration function that must be quantified to obtain the correlation between isotopes in the environment and those in tissues (Bowen et al., 2005; Caut et al., 2009). With this approach, one can hypothetically convert global or continental isoscapes into tissue-specific isoscapes. Establishing environmental isoscapes and then establishing a consistent transformation function that can be applied to an individual or population requires a large amount of sample collection and laboratory analysis prior to the actual application to trace tissue origins (Hobson & Wassenaar, 2019). However, when a tissue (e.g. feathers, wings, claws) is sampled for a certain isotope abundance and the calibration/transformation function is known, one can determine where the tissue was likely generated and subsequently where the organism
was when this tissue originated (Flockhart et al., 2013; Lourenco et al., 2015; Bowen & West, 2019).

A good example of the derivation of a calibration function linking environmental water $\delta^2H$ and animal tissues was that established between wing chitin $\delta^2H$ ($\delta^2H_{\text{wing}}$) of three species of dragonflies (Aeshna interrupta, Aeshna umbrosa and Pachydiplax longipennis) and the mean annual $\delta^2H$ value of the precipitation in the environment ($\delta^2H_{\text{precip}}$) (Hobson et al., 2012). This calibration function was then applied to describe natal origins of common green darners (Anax junius) captured in Texas (Summarized in Figure 1.1).

![Figure 1.1 Simplified protocol for generating a tissue-specific isoscape between $\delta^2H_{\text{wing}}$ and $\delta^2H_{\text{precip}}$ of dragonflies (Aeshna interrupta, Aeshna umbrosa and Pachydiplax longipennis) to determine the potential origins of common green darners samples (Anax junius). (Hobson et al., 2012). This function was also used in a later study on common green darners (Hallworth et al., 2018).](image)

### 1.2.1 Choice of tissue

When animals integrate foodweb stable isotopes into tissues, the isotopes can either become fixed (in the case of metabolically inert tissues such as feathers, hair, or nails) or be ephemeral, depending on the elemental turnover rate of metabolically active tissues (Tieszen et al., 1983; Hobson, 1999; Martínez del Rio & Carleton, 2012; Hobson & Wassenaar, 2019). Thus, if an animal is in equilibrium isotopically with a foodweb at one location and moves to a location which is isotopically distinct from that location, it is possible to infer the isotopic composition of the previous location for a period of time depending on the metabolic characteristics (i.e. turnover rate) of sampled tissue (Hobson & Clark, 1992; Rubenstein & Hobson, 2004; Martínez del Rio & Carleton, 2012). Many
different types of tissues can be sampled for isotopic purposes. In birds, the most commonly used tissue is keratin, a protein matrix, commonly found in metabolically fixed tissues such as feathers, beaks, and claws (Bearhop et al., 2003; Lourenco et al., 2015). For insects, wing chitin (complex carbohydrate-based structure) similarly provides a convenient metabolically inactive tissue in studies of migration (O’Brien et al., 2005; Hobson et al., 2019a).

Isotopic information is not limited to just inferring a location but can be used to determine many other factors, such as timing of movements or residency time, environment quality, and diet shifts (e.g. Sorensen et al., 2009; Heady & Moore, 2012; Reuter et al., 2016). Proteins in blood and muscle are metabolically active tissues that are commonly used in animal movement studies (Evans-Ogden et al., 2004). Differences in the migration timing due to wintering environment quality of American Redstarts (Setophaga ruticilla) and the subsequent effect on arrival times was explored using blood and muscle δ¹³C values (Marra et al., 1998). Birds arriving later at breeding grounds originated from poorer environmental conditions (i.e. more xeric conditions) and subsequently had lower reproductive success and fitness (subsequently studied in Gonzalez-Prieto & Hobson, 2013). That study used both habitat isotopic values on the wintering grounds and tissue isotopic values in arriving male American Redstarts to show that winter habitat quality carried over to influence reproductive success through timing of migration. That key finding was made possible by isotopically examining the appropriate tissues in arriving birds that still retained information from a previous period and was based on knowledge of tissue turnover rates in metabolically active tissues. (Marra et al., 1998).

1.3 Carbon

1.3.1 Carbon isotopes in the environment

One of the most abundant elements found in the natural world is carbon. Though carbon with an atomic mass of 12 is most common (98.89%), a heavier stable isotope, ¹³C, is also present (1.11%) (Wassenaar, 2019). Carbon isotopes are fixed in biotic systems through primary production in plants and the photosynthetic pathway used results in
differences of $\delta^{13}$C values in plant tissues (Park & Epstein, 1961; Smith & Epstein, 1971). All plants use Rubisco (RuBP) as their carbon-fixing enzyme and so RuBP does not cause differences seen in $\delta^{13}$C values of plants using different photosynthetic pathways (C3, C4, and CAM) (Farquhar et al., 1989). Different photosynthetic pathways provide RuBP with an environment varying in partial pressures of CO$_2$ and thus varying concentrations of $^{13}$C for use in photosynthesis (Smith & Epstein, 1971; Farquhar et al., 1982).

C3 plants integrate CO$_2$ directly into the Calvin cycle using a 3-carbon intermediate molecule (O’Leary et al., 1992; Wang et al., 2012). In C4 plants, due to the presence of PEP carboxylase, CO$_2$ is stored as a C4 intermediate molecule in the mesophyll before being shuttled into the bundle sheath, where RuBP is then provided with a higher concentration of CO$_2$ than with C3 plants (Smith & Epstein, 1971; O’Leary, 1989; Wang et al., 2012). Crassulacean acid metabolism (CAM) plants close their leaf stomata temporally as a mechanism that concentrate CO$_2$ levels within the chemical environment of RuBP in xeric environments (Farquhar et al., 1989). With a higher partial pressure of CO$_2$, the probability of integrating $^{13}$C during photosynthesis is higher and thus pathways that allow for CO$_2$-concentrating mechanisms are enriched in $^{13}$C (Farquhar et al., 1982; O’Leary et al., 1992; Wang et al., 2012). C4 plants are typically more isotopically positive (-7 to -15‰) while C3 plants are more negative (-20 to -35‰; Farquhar et al., 1989; O’Leary, 1989). C4 and CAM plants are typically more isotopically similar while C3 plants are more negative than CAM plants, but CAM plants tend to have a more variable range (Farquhar et al., 1989; Nobel, 2009). When water stressed, C3 plants close their stomata, trapping $^{13}$C in the leaves and forcing the plant to utilize more $^{13}$C than before, resulting in their isotopic values being higher than under normal conditions (Farquhar et al., 1982). Seasonality and environmental conditions to which plants are exposed, such as water stress, can affect the values of $\delta^{13}$C, but these variabilities can be modeled (Still et al., 2003). From the information stated previously about how plants typically behave in terms of carbon isotopes, spatial information related to different C4, CAM, and C3 biomes can be modelled in $\delta^{13}$C plant isoscapes (Bowen & West, 2019). One such isoscape that has been extrapolated from both lab experiments and satellite imagery of plant distribution is depicted below (Suits et al., 2005). This isoscape can be
used as a base environmental isoscape for any further manipulations such as generating a tissue specific isoscape for nutrient tracing.

Figure 1.2 Mean δ^{13}C (‰) of terrestrial plants as generated by Suits et al., 2005. Values were generated from an 11-year simulation. Use of carbon isotopes in migratory studies.

Carbon stable isotopes can be used to determine the plant biome origin of tissues (C4, C3 and CAM biomes) as the δ^{13}C values in the environment are determined by primary producers (Farquhar et al., 1982; Kays & Feranec, 2011). Carbon isotopes can also be used alongside other isotopes to constrain potential origins. Commonly, stable carbon and nitrogen isotopes of tissues are used to reconstruct foodwebs by establishing trophic positions and movement from marine compared to terrestrial food sources (Kelly, 2000; Middelburg, 2014). Stable carbon isotopes in combination with stable hydrogen isotopes have been used many times to successfully track organisms at local or continental scales (Hobson, 1999). Though hydrogen isotopes vary at a larger latitudinal scale, carbon isotopes can be used to constrain locations at a specific latitude (Hobson, 1999; Wassenaar & Hobson, 1998).
In an early study, Chamberlain et al. (1997) measured $\delta^{13}C$ and $\delta^2H$ values in the feathers of the Black-throated Blue Warbler (Setophaga caerulescens). The researchers used both isotopes to constrain origins of this species captured on wintering grounds in the Caribbean. In another study, researchers aimed to determine the origin of a true armyworm moth (Mythimna unipuncta) population that migrated to southern Ontario using a multi isotope ($^{13}C$ and $^2H$) analysis approach (Hobson et al., 2018). It was shown through $\delta^{13}C$ analysis of wing chitin that spring immigrants in Ontario were from exclusively C3 biomes. This information would not have been easily determined using traditional extrinsic markers due to the general constraints with body size and therefore the importance of intrinsic methods like isotopes can be clearly seen.

1.4 Hydrogen

1.4.1 Hydrogen isotopes in the environment

The most abundant isotope of hydrogen is $^1H$ (99.98%) followed by deuterium or $^2H$ (0.01%) (Wassenaar, 2019). Hydrogen isotopes move through the environment according to the hydrological cycle and are predominantly traced through precipitation patterns (Dansgaard, 1964; Gat, 1996). This enables researchers to infer $\delta^2H$ in environmental waters in different regions or conditions.

The main principle that drives the distribution of deuterium in the hydrological cycle is Rayleigh distillation (Dansgaard, 1953). Rayleigh distillation is a process that describes the evaporation of water in an open system involving the preferential loss of isotopically lighter water as vapour, resulting in the vapour being relatively depleted in $^2H$ and the environmental source water more enriched in $^2H$ (Dansgaard, 1964; Clark & Fritz, 1997). This process explains the lighter isotopic composition of clouds compared to the oceans or surface waters from which the clouds were formed (Clark & Fritz, 1997). During condensation, depending on ambient temperature, the heavier water vapour molecules condense first and produce rainfall resulting in the remaining (cloud) vapour being more depleted in $^2H$ (i.e. lower $\delta^2H$ values). Progressive rainout from the source results in more and more depleted rainfall overtime (Gat, 1980; Vander Zanden et al., 2015). One factor that can cause values to deviate from the general patterns of Rayleigh distillation is the
continental effect, where precipitation $\delta^2H$ values change more dramatically inland than predicted by Rayleigh distillation due to large temperature gradients (Clark and Fritz, 1997). Another factor that can cause this deviation is the altitude/latitude effect, wherein vapour at increased altitude/latitude being cooler leads to a decrease in vapour $\delta^2H$ compared to lower altitudes/latitudes (Dansgaard, 1964; Windhorst et al., 2013). Though there are multiple sources of variations in precipitation $\delta^2H$ (Wassenaar et al., 2011), many have been well studied and accounted for through the International Atomic Energy Agency - Global Network of Isotopes in Precipitation (IAEA-GNIP) monitoring system, where precipitation $\delta^2H$ was being measured continuously for many years (Gröning, 2004). From the collected data precipitation isoscapes like the one depicted below (Terzer et al., 2013) can be generated. This can be used as a base environmental isoscape for any further manipulations such as generating a tissue specific isoscape for migration studies.

Figure 1.3. Mean growing season $\delta^2H$ in precipitation as depicted by Terzer et al., 2013. Growing season was defined as all months where mean air temperature was greater than 0°C.
1.4.2 Use of hydrogen isotopes in migratory studies

Animals derive their hydrogen isotopes in tissues ultimately from the plants that support the food web and those plants derive their hydrogen isotopes from precipitation (Estep & Dabrowski, 1980; Hobson & Wassenaar, 1999). Animals also obtain hydrogen isotopes from drinking water (Estep & Dabrowski, 1980; Hobson & Wassenaar, 1999). Ultimately, hydrogen in diet and drinking water come from precipitation (Bowen & West, 2019). The δ²H in metabolically inactive tissues like feather keratins are closely linked with environmental water δ²H isoscapes (e.g. Chamberlain et al., 1997; Lott et al., 2003; Hobson et al., 2014). Feathers can thus provide environmental information on the location where tissue synthesis took place and this information is held fixed/inert over time. An inert tissue used to track insects using δ²H isoscapes is wing chitin, as it integrates isotopic information from the origin of synthesis (i.e. the natal site) regardless of other locations visited by the insects (e.g. Hobson et al., 2012; Hobson et al., 2018).

The behaviour of hydrogen isotopes in metabolically active tissues is inherently more complex and the use of δ²H measurements in such tissues is less common due to the potential for hydrogen isotopic exchange (Schimmelmann, 1991; Hobson & Wassenaar, 2019). Biochemically, during the incorporation of hydrogen isotopes into macromolecules that form tissues, there are many opportunities for hydrogen isotopes to undergo isotopic exchange with the environment (Wassenaar & Hobson, 2000; Bowen et al., 2005). This is because hydrogen-oxygen and hydrogen-nitrogen bonds are relatively weak compared to hydrogen-carbon bonds, providing an opportunity for hydrogen isotopes of body water or other sources to exchange with the weaker bound hydrogen isotopes in precursor molecules, including lipid precursors (Sessions et al., 1999). Due to this processing, δ²H can vary inconsistently from the nutrient source, making a calibration or transformation factor difficult to establish especially in lipids (Meier-Augenstein et al., 2013). However, if a calibration relationship linking δ²H in selected tissues with those in the underlying (foodweb or water) isoscape supporting the animal is distinguishable, theoretically such tissues can provide information on provenance (Vander Zanden et al., 2015, 2016).
Hydrogen in metabolically active tissues can theoretically be used in conjunction with other isotopes to establish isotopic changes due to movement and gain information on the diet, timing, stopover, and allocation patterns as long as the isotopic environmental endpoints are known (Phillips & Eldridge, 2006). For an example, in one study $\delta^2$H in muscle of wintering Lesser Snow Geese (*Chen caerulescens*) was used, along with $\delta^{13}$C and $\delta^{15}$N values, to pinpoint the winter origins of migrating Lesser Snow Geese (Hénaux *et al.*, 2012). Muscle is a metabolically active tissue and with information on rate of turnover the origins of the geese were pinpointed to four distinct locations (Hénaux *et al.*, 2012). This information would be difficult to obtain by conventional means because of the difficulty in tracking sufficient numbers of individuals from known winter to known stopover sites where they could be captured and sampled.

### 1.5 Lipids

#### 1.5.1 Lipids and migration

Lipids are abundant macromolecules that are an essential source of energy for migratory organisms (Beenakkers, 1969; McWilliams *et al.*, 2001, 2004). Lipids are commonly found as fatty acids, generally composed of hydrophobic carbon-hydrogen tails attached to a hydrophilic head (Subramaniam *et al.*, 2011; Wang *et al.*, 2019). Lipids can be found throughout the body but also are stored in distinct deposits such as lipid bodies in insects (Kilby, 1963) or fat depots such as the furcular hollow in birds (Blem, 1976; Deppe *et al.*, 2015). Migratory organisms use lipids as the primary fuel source as it carries the highest amount of energy per unit of mass (Beenakkers, 1969; McWilliams *et al.*, 2004). Some fatty acids are conserved during flight for purposes such as reproduction in migratory mixed-strategy breeders (Thomas & George, 1975; Egeler & Williams, 2000) making lipids a unique source for isotopic studies as they are both stored and metabolized. In contrast, income breeders use, for reproduction, nutrient stores such as lipids that are generated (in majority) at or near breeding sites themselves and thus can be used to establish recently visited sites prior to arrival (Morrison & Hobson, 2004; Bond *et al.*, 2007).
To date, little research has been conducted on tracing the nutrient and environmental source of lipids stored as fuel and/or reserves in migrating organisms especially through isotopic means with the goal of generating isoscapes. Lipids are composed primarily of carbon and hydrogen and play a strong role in the migratory success of most organisms, making lipids theoretically amenable to tracing using $\delta^{13}$C and $\delta^{2}$H measurements for purpose of tracking migration (Subramaniam et al., 2011; Wassenaar & Hobson, 2003; Wang et al., 2019). However, there can be a number of isotopic complications related to lipid synthesis that can lead to high levels of discrimination (DeNiro & Epstein, 1977; McConnaughey & McRoy 1979).

1.5.2 Lipid synthesis

When an organism consumes lipids, they enter the body and are routed with little processing prior to storage, leaving little chance for isotopic discrimination (e.g. Podlesak & McWilliams, 2007). When lipids are generated from other nutrients, complex formation processes and metabolic routing leads to isotopic discrimination and isotopic exchange (Sessions, et al., 1999; Liu et al., 2016; Soto et al., 2017). The process of *de novo* lipid synthesis extensively discriminates against heavier isotopes ($^{13}$C and $^{2}$H), rendering lipids more isotopically depleted than other body tissues (DeNiro & Epstein, 1977; McConnaughey & McRoy, 1979). Lipid precursor molecules are also less stable and thus have high potential for isotopic exchange (Fan et al., 2013). Lipid synthesis from other carbohydrate sources (Summarized in Figure 1.2) is relatively conserved amongst various taxa and begins with the glycolysis of glucose (Crabtree & Newsholme, 1975). The primary product from this catabolism, pyruvate, is then converted to acetyl-CoA and eventually forms fatty acids through various additional reactions (Laliotis et al., 2010). Fractionation can occur at various stages in *de novo* lipid synthesis causing a depletion in heavier isotopes in lipids (Melzer & Schmidt, 1987).
Figure 1.4 Simplified process of generating fatty acids from glucose and the interconnected system of nutrient catabolism.

1.5.2.1 Lipid synthesis in Lepidoptera

Insects can store lipids directly from dietary sources or synthesize them from plant carbohydrates (Kozhantshikov, 1938). In lepidopterans, digestive amylases and glycosidases (Droste & Zebe, 1974) initially breakdown carbohydrates being consumed. Following digestion in the midgut, carbohydrates are transferred to the haemolymph (Bushman et al., 2009). Digested carbohydrates are commonly converted to glycogen and trehalose by glycogen synthase and trehalose-6-phosphate synthase, respectively, both of which are present in the lipid body (Bushman et al., 2009; Mattila & Hietakangas, 2017). For lipid synthesis, dietary carbohydrates undergo glycolysis to produce pyruvic acid and ultimately acetyl-CoA (Mattila & Hietakangas, 2017). Trehalose is also converted into forms of glucose (e.g. glucose 6-phosphate) via α, α-trehalase (Sacktor, 1970; Mattila & Hietakangas, 2017). Glucose 6-phosphate is a product of the second stage of glycolysis and glycolysis will produce pyruvic acid and ultimately acetyl-CoA (Crabtree & Newsholme, 1975). Acetyl-CoA then undergoes a series of reactions to produce palmitic acid (Bailey, 1975; Gilbert, 1967) through a cytoplasmic fatty acid synthetase system (a protein bound cytoplasmic enzyme complex) (Thompson et al., 1975; Laliotis et al., 2010). Note that additional systems are responsible for the elongation and desaturation of these de novo synthesized fatty acids to generate the variety of fatty acids (commonly triacylglycerol) found in the true armyworm moth (Downer, 1978). With such a complex
synthesis process there are many opportunities for isotopes to be integrated in or selected against. For example, acetyl-CoA synthesized with $^{13}$C instead of $^{12}$C is less likely to be integrated into the Krebs Cycle (Blair et al., 1985). The enzyme trehalase is a hydrolase (Sacktor, 1970), and thus acts as a source of hydrogen isotope incorporation, which can contribute to hydrogen isotopic discrimination and potential exchange.

### 1.5.2.2 Lipid synthesis in birds

When a bird ingests carbohydrates, the process of digestion is similar to that found in insects. Carbohydrates are primarily broken down into glucose using various amylases (Droste & Zebe, 1974; Karasov & Martinez del Rio, 2007) in preparation for glycolysis. Glycolysis provides pyruvate that gets converted into acetyl-CoA using pyruvate dehydrogenase, an enzyme known for discriminating against $^{13}$C and $^2$H (DeNiro & Epstein 1977; Melzer & Schmidt 1987). Lipogenesis occurs primarily in the liver (Pearce, 1980; Zaefarian et al., 2019), where acetyl-CoA is converted via a series of reactions (Richards et al., 2003) into basic fatty acid units. These fatty acids are commonly further processed via elongation, branching or attachment to glycerol through many enzymes, such as thioesterases (Klasing, 1998), generating triacylglycerides. Triacylglyceride is the most common form of body lipid in birds for storage in various fat body deposits (Blem, 1976; Deppe et al., 2015).

During lipid mobilization in birds most of the lipid being used as fuel comes from the various fat stores in the body, including the furcular hollow (Blem, 1976). Lipids are transported as non-esterified fatty acids and converted back into acetyl-CoA using various enzymatic processes (summarized as B oxidation) (Weber, 1992). Acetyl-CoA then enters the Krebs cycle to produce ATP, CO$_2$ and water. The CO$_2$ and water are released from the organism during respiration. Breath CO$_2$ has been used in many experiments to trace source substrates of metabolic fuel and nutrient allocation patterns (Voigt et al., 2008; Engel et al., 2009 Welch et al., 2016). This measurement tool is not static and can be used to explore fuel use pattern changes over a period of time (e.g. Hatch et al., 2002; McCue et al., 2013). These applications are possible due to the fact that when proteins, lipids or carbohydrates are being used, the interconnection point of metabolism, acetyl-CoA, will generate the same by-products (CO$_2$ and H$_2$O). Thus, if
source fuel $\delta^{13}C$ and $\delta^2H$ values are known, the breath CO$_2$ and water vapour can be traced back isotopically.

1.6 Objectives and thesis organization

This thesis explores the capacity of using $\delta^2H$ and $\delta^{13}C$ measurements of lipids as a viable tracking tool to infer nutritional sources in migrating birds and insects. This will require an investigation of the viability of producing species-specific “liposcapes” or predictable, spatially-explicit lipid isotope patterns through the establishment of diet-to-lipid isotopic relationships (calibration functions) in each taxon. If successful, this study will form the foundation of an important tracking tool for ecologists and ecophysicologists.

For insects, the primary model organism was the true armyworm (*Mythimna unipuncta*) as it is a seasonal migratory insect with both shorter and longer north-south, cross-continental migration patterns (Guppy, 1961). Armyworm adults feed on nectar, which can be easily manipulated isotopically (using C3 and C4 sugars and deuterium-spiked waters). True armyworm moths can be induced to put on lipid stores rapidly in captivity (Benoit, 2017). Isotopic discriminations between nectar and stored lipids for $\delta^2H$ and $\delta^{13}C$ were explored to investigate the possibility that the connectivity seen in proteins is also found in lipids. It was predicted that a linear isotopic calibration function would be established between dietary treatments and *de novo* synthesized body lipids for both $\delta^2H$ and $\delta^{13}C$ (as seen with other insect studies, e.g. Hobson *et al.*, 2012 and Hobson *et al.*, 2018) (see Chapter 2).

In addition, the traceability of different diets through the use of stable isotopes found in *de novo* synthesized lipids was explored through an isotopic diet switching experiment. This can be used to infer fueling rates and determine arrival times and stopover duration by reflecting changes in dietary uptake (e.g. Paxton & Moore, 2017). I also predicted that a rapid rate of isotopic uptake during lipid acquisition will be seen through diet switching between isotopically distinct diet sources for both $\delta^2H$ and $\delta^{13}C$ (see Chapter 2).
The primary model organism for birds was the White-throated Sparrow (*Zonotrichia albicollis*), as it is a short-distance migrant in North America with a broad diet (Falls & Kopachena, 2010). This species can be kept and manipulated easily in captivity without changes in behaviour, especially in response to diet changes (Pierce & McWilliams, 2004). Sparrows were provided with isotopically distinct diets (varying in both $\delta^2$H and $\delta^{13}$C) in order to explore the isotopic relationship between dietary sources and *de novo* synthesized lipids. It was hypothesized that body lipid $\delta^2$H and $\delta^{13}$C values would be strongly correlated with those in dietary substrates. Based on previous literature on isotopic discrimination (i.e. Hobson *et al.*, 1999; Tonra *et al.*, 2015), I predicted a linear relationship between lipid $\delta^2$H and $\delta^{13}$C values and the mean dietary $\delta^2$H and $\delta^{13}$C values from which lipids were formed (see Chapter 3).

Additionally, the isotopic sources of breath CO$_2$ and water vapour were explored. Sparrows were fasted or fed, and breath metabolites (CO$_2$ and water vapour) assessed through $\delta^2$H and $\delta^{13}$C to establish the potential of tracing lipid use and subsequently dietary sources of lipids through the by-products generated from metabolic catabolism. I predicted that fasted birds would have isotopically depleted breath CO$_2$ and water vapour compared to fed birds reflecting the use of lipids when dietary sources are unavailable (based on previous literature, e.g. McWilliams *et al.*, 2004 and McCue *et al.*, 2013). I also predicted that $\delta^2$H and $\delta^{13}$C values of water vapour and CO$_2$ would reflect the isotopic distinctions of the dietary treatments (see Chapter 3).

My MSc. thesis is organized in an integrated article format with two data chapters (described above). In Chapter 1, I discussed the importance of addressing current changes in migration patterns and how stable isotopes can be used as an intrinsic tool to study migration through nutrient and tissue sourcing. I also examined the distribution and use of both $\delta^2$H and $\delta^{13}$C in biological studies as well as the potential lipids have in providing information regarding origins, fuelling and stopover. In Chapter 4 the overall findings of Chapter 2 and Chapter 3 are summarized and further avenues for my research study topic are suggested.
1.7 References


Chapter 2

2 Tracing nutrient sources to lipids in insects using stable isotopes ($\delta^{13}C$, $\delta^2H$)

2.1 Introduction

Migration is a phenomenon that occurs in species across various taxa and is driven by seasonal changes in resource availability (Dingle, 2014). Although less appreciated compared to migratory vertebrates, migration is common in insects (Holland et al., 2006) and their movements at continental scales is impressive (Chapman et al., 2015). Such movements can be achieved by powered or gliding flight, but the use of high-altitude winds often facilitates long-distance movements, which are commonly multi-generational (Anderson, 2009; Stefanescu et al., 2013).

Timing of migration in nectivorous insects often coincides with the seasonal availability of nectar resources (Holland et al., 2006; Stefanescu et al., 2013). Physiological and behavioural mechanisms have evolved that optimize migration in insects, which involves periodic refuelling at key stopover sites or stages of the life cycle (Chapman et al., 2015). Key to understanding the life history of migrating insects is evaluating connections between sites used for lipid synthesis and ultimate destinations. Establishing spatial or geographic origins of macronutrients like lipids in migratory fauna is daunting. The use of intrinsic markers, such as naturally occurring stable isotope ratios in biological tissues, has become one tool to track origins of migrating animals (Hobson et al., 2019) largely through isotopic measurements of metabolically inactive tissues like keratins or chitins (e.g. Bearhop et al., 2003; Hobson et al., 2019). It is possible that a similar approach could be used to infer spatial origins of endogenous macromolecules such as stored lipids. Like many migratory organisms, insects fuel migratory flights through lipids that can be stored endogenously (Beenakkers, 1969). Lipids can also be used by insects to buffer against periods of fasting (McCue et al., 2015). Essential to successful insect migration or survival, then, is the seasonal or periodic accumulation of lipids.

Spatial isotopic patterns or “isoscapes” have been used to trace movements of migratory animals. Researchers typically use metabolically inert tissues like keratins and chitins that
form feathers, hair, and insect wing tissues (e.g. Alisauskas et al., 1998; Hobson et al., 2018). In particular, stable hydrogen isotope ratios ($\delta^2$H) in animal tissues have been useful because the distribution of this isotope is closely linked with continental patterns of $\delta^2$H in precipitation (Wassenaar & Hobson, 1998) and such patterns are passed through the food web to the consumer. Other elements such as carbon have stable isotope ratios ($\delta^{13}$C) that can be used to infer origins as well as diets of migratory animals (Hobson et al., 2012; Adams et al., 2016). The approach to using stable isotope ratios in animal tissues to determine their origins requires a means of linking stable isotope values associated with an isoscape (for example a precipitation $\delta^2$H surface) with isotopic values found in tissue. The arithmetic change ($\Delta$), also known as discrimination, between substrate and tissue isotope values, is a transformation function or calibration function that must be quantified to obtain the correlation between isotopes in the environment and in tissues (Wunder, 2012). When the calibration function is established, one can hypothetically convert known continental isoscapes into tissue-specific isotopic maps for a given taxon (Bowen & West, 2019; Hobson et al., 2019). I speculated that because lipids are composed primarily of carbon and hydrogen, they could potentially contain spatial information on the origins of where individuals accumulated lipids. Of particular utility would be those lipids that are stored in fat bodies and which are expected to be metabolically (and hence isotopically) inert up to the point of being metabolized (Arrese & Soulages, 2010).

A first step in evaluating the potential for stable isotope measurements to provide information on the provenance of nutrients leading to stored lipids is a demonstration of predictable linkages between environmental isotopic signals and the lipids produced at those sites. Animals uptake environmental isotopic signals by processing dietary macromolecules into metabolically active and inactive tissues (Bowen & West, 2019; Hobson et al., 2019). The degree of isotopic change between environmental substrates and synthesized tissue will depend upon the number of steps involved where isotopic discrimination can take place (DeNiro & Epstein 1977). Metabolic processing of diet carbohydrate into the moth lipids results in isotopic discrimination and subsequent changes in $\delta^2$H and $\delta^{13}$C values of lipid compared to diet (DeNiro & Epstein 1977; Chamberlain et al., 2004). Plant-based carbohydrates have to be ingested by insects and
then mobilized to undergo a breakdown into their basic monomers, such as glucose, for lipid synthesis to occur. Ingested carbohydrates are broken down enzymatically in preparation for absorption and then passed into the hemolymph to be deposited in the lipid body as trehalose or glycogen (Sacktor, 1970; Bushman et al., 2009). When in excess, dietary carbohydrates and trehalose will be catabolized and enter glycolysis, which produces pyruvic acid (Crabtree & Newsholme, 1975) and the conversion of pyruvic acid into acetyl-CoA acts as the interconnection point between carbohydrates and lipids. Evidently, lipid synthesis in insects requires various steps with various enzymes. With such a complex biochemistry, it has not been clear previously how well lipid isotope values can be consistently linked to the environments where they were produced.

I investigated the use of $\delta^{2}$H and $\delta^{13}$C values in insect lipids as a means of identifying origins of these elements from diet. I studied a captive population of the true armyworm (*Mythimna unipuncta*), which was amenable to dietary manipulations. This moth is a migrant insect that arrives in southern Ontario in the spring but is unable to overwinter (Guppy, 1961). Emergent moths maintain some lipid from larval stages, but adults are known to synthesize and store large amounts of lipids to fuel migration and reproduction (Kilby, 1963; Levin et al., 2017). My objectives were to investigate if $\delta^{2}$H and $\delta^{13}$C values in adult nectar diet reflected those in subsequently synthesized lipids. Using nectar that had been manipulated isotopically to reflect different sugar ($\delta^{13}$C) and water ($\delta^{2}$H) isotopic values, I sought to quantify the isotopic composition of lipids and evaluate the potential for using this isotope approach to derive information on geographic origins of dietary nutrients. Discrimination of $\delta^{13}$C is explored between the carbohydrate source and *de novo* synthesized lipids. Experimental water treatments were used to establish the integration of body water into lipid. However, in nature, water and carbohydrate sources that comprise nectar are expected to be isotopically similar as they are synthesized from a single environmental water source driving the $\delta^{2}$H value of the plants (Farquhar et al., 2007; Cernusak et al., 2015). For this reason, discrimination was explored using the $\delta^{2}$H values of the carbohydrate (vs. water) source as this is more reflective of the macronutrient driving lipid formation. Two-point calibration functions were generated between lipid and dietary source for both $\delta^{2}$H and $\delta^{13}$C. Calibration relationships have been shown on many occasions to be linear functions (e.g. Wolf et al., 2011; Hobson et
al., 2012) thus, a two point calibration function can be reasonably assumed to summarize the discrimination relationship. I reasoned that my study is a fundamental first step toward ultimately deriving spatially explicit maps of the origins of lipids or “liposcapes”.

2.2 Methods

Larval stage treatment: My experimental colony was derived originally from individuals collected at the Environmental Science Western Field Station near London, Ontario (43.07°N, 81.34°W). Larvae (n=180) were fed a pinto bean (C3) diet (Shorey & Hale, 1965) under standard long day conditions (25 °C, 65% RH and photoperiod of 16L:8D). Once larvae pupated, they were sexed and divided into groups for isotopic discrimination and uptake experiments. In the control group, ten newly emerged males and females were sacrificed via freezing without any exposure to diet. Post-sacrifice, sex was verified again, and the lipid body was excised then extracted for 48 hours with a 2:1 (v:v) mixture of chloroform:methanol. After extraction, lipids were recovered following evaporation in a fume hood for >24 hours (23°C, 101.3 kPa). The remaining insects were divided into two groups, one to evaluate isotopic discrimination between diet and body lipid and the other for isotopic uptake or change in body lipid due to isotopic change in food sources. Both groups were held under standard long day conditions.

Isotopic discrimination: A randomly selected sample of true armyworm moth pupae was sexed and separated into four groups, each with ten females and ten males. Each group was fed a different treatment diet at emergence using a 2x2 design: (1) high $\delta^{13}$C (C4 cane sugar) with high $\delta^2$H (spiked) water, (2) high $\delta^{13}$C with low $\delta^2$H (tap) water, (3) low $\delta^{13}$C (C3 maple sugar) with low $\delta^2$H water and (4) low $\delta^{13}$C with high $\delta^2$H water. Solutions were made by adding 450g of sugar to a 1000 ml volumetric cylinder and filling the rest of the cylinder with spiked or unspiked water, so solutions were controlled at 45% concentration, which falls within the range of ideal concentrations for moths to take up nutrients efficiently (Benoit, 2017). Deuterium spiked water was prepared by adding a quantity of 99.9 atom % heavy water (deuterium oxide) (Sigma Aldrich) to be approximately +718 ‰ compared to the unspiked (London, Ontario) tap water that was approximately -55 ‰ (Table 2.1).
Table 2.1 Average isotopic (δ^{13}C and δ^{2}H) values of diet and dietary components of synthetic moth nectar used in my experiment. S refers to “spiked” water and U to “unspiked” (tap) water. Sample size is 3 in all cases.

<table>
<thead>
<tr>
<th>Diet Component</th>
<th>^{13}C Mean ± SD (%)</th>
<th>^{2}H Mean ± SD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3/S</td>
<td>-25.15 ± 0.06</td>
<td>97.61 ± 47.22</td>
</tr>
<tr>
<td>C3/U</td>
<td>-25.13 ± 0.04</td>
<td>-24.74 ± 2.24</td>
</tr>
<tr>
<td>C4/S</td>
<td>-12.18 ± 0.03</td>
<td>208.55 ± 15.06</td>
</tr>
<tr>
<td>C4/U</td>
<td>-12.23 ± 0.06</td>
<td>6.53 ± 16.19</td>
</tr>
<tr>
<td>C4 sugar</td>
<td>-12.17 ± 0.05</td>
<td>-12.17 ± 1.21</td>
</tr>
<tr>
<td>C3 syrup</td>
<td>-25.17 ± 0.03</td>
<td>-28.05 ± 8.57</td>
</tr>
<tr>
<td>Spiked water</td>
<td>--</td>
<td>718.03 ± 9.02</td>
</tr>
<tr>
<td>Unspiked water</td>
<td>--</td>
<td>-55.17 ± 2.04</td>
</tr>
</tbody>
</table>

After the sixth day of feeding, insects were sacrificed via freezing and lipid bodies were excised. Carbon isotopic discrimination (Δ^{13}C) is based on the isotopic δ^{13}C difference between dietary sugar and lipids. Hydrogen isotopic discrimination (Δ^{2}H) is more complex and arises from H-exchange occurring between insect body water and insect lipid precursors as well as from δ^{2}H differences between insect dietary sugar and insect lipids. Here, for water and carbohydrate sources I focused on δ^{2}H discrimination associated with carbohydrates. I reasoned 1) from a mass balance perspective, plant carbohydrates in the form of nectar represents the major source for lipid synthesis (Arrese & Soulages, 2010) and thus hydrogen isotopic contribution to lipids and 2) that insect body water would be closely linked to environmental water δ^{2}H driving the foodweb (Hobson et al., 1999b; Bowen & West, 2019).

Isotopic uptake: Randomly selected pupae were sexed and separated into two groups, each containing 20 males and 20 females. The first group was used to examine uptake in δ^{2}H and the second group in δ^{13}C. Solutions were the same as those made for the (above) isotopic discrimination experiment. The first group was fed a low δ^{2}H and high δ^{13}C
sugar solution for the first day of emergence, after which the diet was switched to a high δ²H water but unchanged δ¹³C source (C4 cane sugar) for another three days. In the other group, 20 males and 20 females were raised on low δ²H water and high δ¹³C sugar for the first day of emergence as before. Following this, the diet was switched to a low δ¹³C (C3 maple sugar) solution but an unchanged δ²H water for another three days. Five males and 5 females were sacrificed via freezing at the beginning of days 1, 2, 3, and 4 in each group. Following the sacrifice, lipid bodies were excised. In the first treatment group the change in δ²H of moth lipid was evaluated daily over the duration of the experiment while the change in δ¹³C of moth lipid value was recorded daily in the second treatment group.

**Stable Isotope Measurements:** For δ²H analyses, 0.35mg of lipid were loaded into silver capsules and crushed. These samples were loaded into a uni-prep (Eurovector; Milan, Italy) carousel at room temperature, evacuated and kept under a pressurized He atmosphere. Samples were directly introduced into a Eurovector 3000 elemental analyser and combusted pyrolytically at 1350 °C on glassy carbon. Resultant gases were separated and introduced via a ConFlo interface to a Thermo Delta V Plus stable isotope mass spectrometer (Thermo Instruments, Bremen, Germany). All tissue samples were calibrated relative to silver encapsulated water USGS standards (VSMOW2: 0 ‰; USGS 46: -235.8 ‰). Within-run standards also included CBS (-197 ‰) and KHS (-54.1 ‰) keratin standards to adjust for instrument drift. Based on within-run replicates of standards, I estimated measurement error to be ± 3 ‰.

Water δ²H analyses were performed using an Off Axis Integrated Cavity Output Spectroscopy (OA-ICOS) using a Los Gatos Research DLT-100 laser spectrometer (Mountain view California). I used two calibrated reference waters (INV1 δ²H = -217.7 ‰ and ROD3 δ²H = -3.9 ‰, respectively) to normalize raw delta values to the VSMOW–SLAP scale. To minimize memory effects, samples and reference waters were injected 9 times and the last 5 measurements were averaged to obtain the final raw delta values. Precisions as determined by replicate analyses of samples and reference waters were ± 1 and 0.1 ‰ respectively.
For $\delta^{13}$C measurements, approximately $0.35 \pm 0.02$ mg of samples were weighed into 4 x 3.2 mm tin pressed capsules. Concurrent $\delta^{13}$C and $\delta^{15}$N analyses were performed using a Costech Elemental Analyzer coupled to a Thermo Delta Plus XL isotope ratio mass spectrometer (Thermo Instruments, Bremen, Germany) operated in continuous flow mode with helium carrier gas, but only $\delta^{13}$C values are reported here. Two standards, USGS-40 and USGS-41, were included for every ten samples, and two internal laboratory standards, powdered keratin (MP Biomedicals Inc., Cat No. 90211, Lot No.9966H) and IAEA-CH-6 were included to monitor instrument drift and provide a check on accuracy over the course of each analytical session. Values of $\delta^{13}$C were calibrated to VPDB using USGS-40 ($\pm 0.1$ ‰ 1 SD, $n = 8$, accepted $\delta^{13}$C = -26.4 ‰) and USGS-41 ($\pm 0.1$ ‰, $n = 8$, accepted $\delta^{13}$C = +37.6 ‰). Measurement error was $\pm 0.1$ ‰ for $\delta^{13}$C.

**Statistical Analysis:** All statistical tests (ANOVA, Kruskal Wallis tests, and GLMS) were performed using R studio (version 3.4.2 (2017-09-28)) as required. Graphs were generated using Microsoft Excel 2010. ANOVA was used to compare lipids under differential diet treatments or days of uptake where normalized data were collected. When the data sets were not normal GLMs and Kruskal Wallis tests were performed.

### 2.3 Results

Lipid $\delta^{13}$C values measured in this study showed clear statistical differences that were reflective of differences in C3 and C4 diets provided (GLM, df =4.81, $F = 165.31$, $P < 0.0001$) (Figure 2.1,Table 2.2).
Figure 2.1 Differences in δ\textsuperscript{13}C values of moth body lipid in relation to differential diet treatments. (*) indicate statistically significant differences in the δ\textsuperscript{13}C value of body lipid on that diet (P<0.05).

Lipid δ\textsuperscript{2}H values measured in this study showed clear statistical differences that were reflective of differences in spiked and unspiked water sources provided (GLM, df=4,81, F = 74.64, P <0.0001) (Figure 2.2, Table 2.2). There was also a statistical difference between the lipid δ\textsuperscript{2}H values generated from the unspiked water sources but differential carbohydrate source (Appendix A, Table A1). There was no effect of sex on the lipid δ\textsuperscript{13}C or δ\textsuperscript{2}H values (Appendix A, Table A1).
Figure 2.2 Differences in $\delta^2$H values of moth body lipid in relation to differential diet treatments. (*) indicate statistically significant differences in the $\delta^2$H value of body lipid on that diet (P<0.05).

Table 2.2 Average isotopic ($\delta^{13}$C and $\delta^2$H) values of adult moth lipids under isotopically distinct diets. S refers to “spiked” water and U to “unspiked” (tap) water.

<table>
<thead>
<tr>
<th>Diet</th>
<th>$^{13}$C Mean ± SD (%)</th>
<th>N</th>
<th>$^2$H Mean ± SD (%)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>-23.57 ± 3.53</td>
<td>8</td>
<td>-298.69 ± 37.56</td>
<td>8</td>
</tr>
<tr>
<td>C3/S</td>
<td>-25.21 ± 2.43</td>
<td>20</td>
<td>-243.07 ± 28.57</td>
<td>20</td>
</tr>
<tr>
<td>C3/U</td>
<td>-26.24 ± 0.17</td>
<td>17</td>
<td>-346.62 ± 19.30</td>
<td>17</td>
</tr>
<tr>
<td>C4/S</td>
<td>-15.98 ± 1.24</td>
<td>19</td>
<td>-226.75 ± 23.74</td>
<td>19</td>
</tr>
<tr>
<td>C4/U</td>
<td>-15.72 ± 1.46</td>
<td>18</td>
<td>-325.28 ± 22.70</td>
<td>18</td>
</tr>
</tbody>
</table>
Based on a constant tap water source, I derived a calibration function linking $\delta^2H$ in moth lipids ($\delta^2H_{\text{lip}}$) to nectar (C3 and C4) carbohydrates ($\delta^2H_c$) as $\delta^2H_{\text{lip}} = 1.34 \times \delta^2H_c - 308.93‰$ (Figure 2.3).

![Diagram showing the relationship between $\delta^2H$ of body lipids and $\delta^2H$ of carbohydrate.](image)

**Figure 2.3** $\delta^2H$ value of body lipids of moths in relation to the $\delta^2H$ value of dietary carbohydrate.

Similarly, for $\delta^{13}C$, using a single tap water nectar source and switching the C3 and C4 carbohydrate component of nectar, I derived a calibration function linking $\delta^{13}C$ in moth lipids ($\delta^{13}C_{\text{lip}}$) to nectar $\delta^{13}C$ ($\delta^{13}C_c$) as $\delta^{13}C_{\text{lip}} = 0.80 \times \delta^{13}C_c - 5.99‰$ (Figure 2.4).
Figure 2.4 $\delta^{13}$C value of body lipids of moths in relation to the $\delta^{13}$C value of dietary carbohydrate.

From this information, a two-endpoint isotopic mixing model was used to determine the proportional contribution of each source, including the contribution of larval lipid $^2$H and $^{13}$C to the lipid isotopic value of adult fed moths. In both $\delta^2$H and $\delta^{13}$C values of adult lipids, larval lipids contributed 19% of total lipid values ($\pm 11\%$ and $\pm 110\%$ for $\delta^{13}$C and $\delta^2$H respectively; Appendix A, Equation A.1 & A.2). In the tap water treatment groups, carbohydrates contributed to the majority of adult $^{13}$C lipid values ($81 \pm 11.0\%$; Appendix A, Equation A.1) and $^2$H lipid values ($68 \pm 101\%$; Appendix A, Equation A.2).

My uptake experiments indicated that adult moth lipid isotope values rapidly grew to reflect those of the dietary nectar source post-emergence (Table 2.3).
Table 2.3 Average isotopic (δ\textsuperscript{13}C and δ\textsuperscript{2}H) values of moth lipids measured for the C4/U to C4/S diet change (change δ\textsuperscript{2}H source) and the C4/U to C3/U diet change (change in δ\textsuperscript{13}C source) experiments. S refers to “spiked” water and U to “unspiked” (tap) water

<table>
<thead>
<tr>
<th>Day</th>
<th>Diet</th>
<th>Mean ± SD (‰)</th>
<th>N</th>
<th>Mean ± SD (‰)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>C4/U</td>
<td>-18.14 ± 1.20</td>
<td>10</td>
<td>-321.03 ± 11.75</td>
<td>10</td>
</tr>
<tr>
<td>2</td>
<td>C4/S</td>
<td>-16.12 ± 0.89</td>
<td>10</td>
<td>-300.85 ± 8.28</td>
<td>10</td>
</tr>
<tr>
<td>3</td>
<td>C4/S</td>
<td>-15.92 ± 0.88</td>
<td>10</td>
<td>-285.11 ± 12.31</td>
<td>10</td>
</tr>
<tr>
<td>4</td>
<td>C4/S</td>
<td>-16.01 ± 0.84</td>
<td>10</td>
<td>-265.23 ± 21.87</td>
<td>10</td>
</tr>
</tbody>
</table>

For the δ\textsuperscript{2}H nectar water uptake experiment, going from tap-water to a spiked-water diet, the best fit model was a logarithmic change ($R^2 = 0.96$, Figure 2.5) with significant changes between Day 1 and the rest of the days (ANOVA, df = 3, F = 26.79, $P < 0.0001$). Differences also occurred between Day 2 and Day 4 (Tukey HSD, $p < 0.0001$) and Day 3 and Day 4 (Tukey HSD, $p = 0.02$) but not between Day 2 and Day 3 (Tukey HSD, $p = 0.08$).
Figure 2.5 δ^2H value of body lipids of moths raised on spike to unspiked switched diets. Dotted line indicates function of best fit. Different letters indicate statistically significant differences in the δ^2H value of body lipid on that day (P<0.05).

Similarly, a logarithmic function best described the uptake of ^13C in lipid following a switch from C3 to a C4 nectar source (R^2 = 0.80, Figure 2.6) where there was a significant change in lipid δ^13C after the first day of feeding (GLM, df = 3,38; F= 11.06, P < 0.0001) and between Day 3 and Day 4 (Kruskal test χ^2 = 4.172, df = 1,19 P = 0.04), but not Day 2 and Day 3 (p = 0.20, Appendix A, Table A.1) or Day 2 and Day 4 (p = 0.16, Appendix A Table A.1).
Figure 2.6 δ\textsuperscript{13}C value of body lipids of moths raised on C4 to C3 switched diets. Dotted line indicates function of best fit. Different letters indicate statistically significant differences in the δ\textsuperscript{13}C value of body lipid on that day (P<0.05).

2.4 Discussion

My study clearly demonstrates that lipids synthesized by adult moths following emergence reflect the δ\textsuperscript{13}C and δ\textsuperscript{2}H values of their adult nectar diet. These results follow on from earlier studies based on δ\textsuperscript{13}C and δ\textsuperscript{15}N measurements that link metabolically active tissues of insects to their diets (O’Brien et al., 2000, 2005; Peters et al., 2012). For moths, lipid synthesis is rapid and fat bodies quickly approach isotopic equilibrium with the local diet during changes the in δ\textsuperscript{13}C and δ\textsuperscript{2}H values of dietary sources. Larval lipids contribute to the adult moth lipids as true army worm moths obtain about 15-20 % (Benoit, 2017) of their adult lipid mass from their larval stage at emergence, which aligns with the proportions established in this experiment (19%). Establishing the proportional contributions to lipid δ\textsuperscript{13}C and δ\textsuperscript{2}H values indicated that dietary sources to lipids can theoretically be traced isotopically. Dietary sources contributing to adult lipids post emergence and larval lipid contributions at least during the early adult stage can also be traced using this approach. Discerning multiple sources of lipids that were synthesized at
various times, (e.g. Levin et al., 2017) could be modeled, should potential dietary input isotope endpoints be known (Moore & Semmens, 2008; Phillips et al., 2014). However, there was a large deviation (± 101%) seen in the estimated proportion of δ²H arising from the carbohydrate source. This variation likely reflected the fact that the carbohydrate δ²H values of the two treatments were very close (i.e. 15.88 ‰ difference). In isotopic sourcing models having dietary source end points that are close can confound estimates of proportional contribution (Phillips et al., 2014; Hobson & Wassenaar, 2019). To increase the accuracy of the proportional contribution of carbohydrate δ²H values to lipid δ²H values carbohydrates with more isotopically distinct δ²H values should be used.

Lipid δ¹³C measurements clearly reflected those of the nectar carbohydrate and can be used to trace the source of feeding corresponding to the plant photosynthetic pathway (C₃, C₄/CAM) or other factors affecting plant δ¹³C values (Farquhar et al., 1989; O’Leary, 1989). Larval lipid δ¹³C was also reflective of a C₃ origin regardless of any metamorphic changes that could have occurred as seen in δ²H values (Chamberlin, 2004) and thus can also be used to trace changes in dietary sources of stored lipids at different life stages of lepidopterans. Values of δ²H are potentially more useful for tracing provenance of lipid synthesis but are also more complicated due to the potential inputs of hydrogen isotopes from both insect diet and body water. Nonetheless, my study provides strong evidence that insect lipids follow both body water δ²H and carbohydrate δ²H and that this isotope will potentially be useful in deriving origins of lipids stored by migrating insects.

Moth lipid δ²H values showed a strong correlation with the carbohydrate and water components of the nectar source from which they were synthesized. Water from the nectar can be incorporated directly and indirectly. The synthesis and disassembly of trehalose requires the direct incorporation of water (Sacktor, 1970) and thus free body water can be integrated at this step. Indirectly, when the lipid molecule is undergoing molecular changes such as being broken apart or synthesized hydrogen isotopes can also be exchanged with hydrogen isotopes present in free body water (Schimmelmann, 1991; Soto et al., 2017). The incorporation of dietary water into lipids is interesting because lipids are composed almost exclusively of carbon-hydrogen bonds that are not subject to
isotopic exchange once formed (Myint et al., 2014). Thus, the hydrogen isotopic exchange with body water occurred at the precursor stage as previously demonstrated in Quail (Coturnix japonica) by Hobson et al. (1999b).

Using isotopic measurement of insect lipids to infer where they were synthesized at continental scales in the manner that has been demonstrated for metabolically inactive tissues such as keratins and chitins (Wassenaar, 2019) is complicated by the fact that insects ingest both plant water and carbohydrates to form stored lipids to fuel migration. If I assume that the δ²H values of these two components of diet are similar and that from a mass balance perspective they will be dominated by plant sugars (Arrese & Soulages, 2010), then the calibration equation I presented here linking δ²H唇 and δ²Hc provides a means of converting growing-season average precipitation δ²H isoscapes into moth “liposcapes”. However, should the water component of nectar differ substantially from that of plant sugars, the calibration of such an isoscape becomes more complex. Currently, there are few data contrasting δ²H values of plant macromolecules and most interest has been focused on cellulose and leaf water fractions (e.g. West et al., 2008, Mora & Zanazzi, 2017). Additionally, the fact that I derived a calibration relationship between δ²H唇 and δ²Hc with a slope greater than one indicates that discrimination factors will change depending on the value of the nectar substrate (see also Peters et al., 2012). This is not necessarily a problem if calibration relationships can be derived by sampling animals across landscapes in the wild vs. raising them in the laboratory where nectar water and carbohydrate fractions can be measured. This has formed the basis for tracking migratory butterflies (Hobson et al., 1999a) and dragonflies (Hobson et al., 2012) through isotopic measurements of wing chitin across large geographic gradients. Other problems associated with controlled laboratory studies is the use of ²H enriched water sources that are outside the natural range experienced by animals (Cong et al., 2010).

Thus, while my study provides an important proof of principle opening up the potential for tracing nutrient origins to lipid synthesis in migratory insects and other animals, I now advocate the sampling of either plant nectar or insect lipids across known latitudinal gradients in precipitation δ²H.
Future research should aim to establish and refine the isotopic connections between environmental waters and the nectars produced by plants. Establishing the $^2$H discrimination relationships between plant nectar and local precipitation in plants would be key in generating moth or other insect “liposcapes” in a similar manner that formed the basis of monarch butterfly wing chitin (Hobson et al., 1999a) or dragonfly wing (Hobson et al., 2012) isoscapes. Finally, for convenience, I used analyses of bulk lipids in moths but readily recognize the potential utility of examining pathways linking individual fatty acids to their source components (McMahon & Newsome, 2019) as a next step in evaluating the traceability of lipids isotopically to dietary sources.
2.5 References


Chapter 3

3 Tracing nutrient sources to lipids in passerines using stable isotopes ($\delta^{13}$C, $\delta^2$H)

3.1 Introduction

Migration plays a vital role in the survival of many organisms as it enables the optimal use of resources over changing seasons (Dingle, 2014). Migratory movement can occur at various spatial scales. In birds, long-distance migratory flight is energy-demanding and requires energy-dense fuel (McWilliams et al., 2004) typically acquired or synthesized at key pre-migratory or stopover sites. Lipids are the optimal fuel facilitating migration and birds can rapidly convert carbohydrate and lipid dietary macronutrients to stored reserves (Odum & Connell, 1956; Pierce & McWilliams, 2005). In addition to fuelling flight, lipids can also be stored as capital for reproduction following arrival to breeding grounds (Hobson et al., 2015). Despite the widely recognized importance of energy loading during migration and stopover, very little is known about the key locations and foodwebs supporting lipid synthesis and storage in migratory birds and other animals. Identifying habitats and foodwebs that facilitate long-distance migration is a key conservation issue (Moore et al., 2007). One approach to this challenge has been the quantitative evaluation of the composition of essential and non-essential fatty acids in animal diets and subsequent fat depots (Egeler et al., 2003; Iverson et al., 2004). This approach has contributed to understanding dietary lipid sources leading to lipid stores in several animals but is not necessarily able to link locations where lipids are synthesized. A promising tool to trace provenance of nutrients leading to lipid synthesis in animals is the measurement of naturally occurring stable isotopes of the two primary elements present in lipids, namely carbon and hydrogen (Subramaniam et al., 2011; Hobson & Wassenaar, 2019). Using stable isotope analyses of these elements in avian metabolically inert (keratinous) tissues like claws and feathers and linking these to known spatial isotopic patterns or isoscapes (Bowen & West, 2019) has proven to be a viable approach to tracking migration and origins of several species (Wassenaar, 2019). Reconstructing sources of endogenous vs. exogenous nutrient allocations to eggs, including their lipids, using stable isotopes of carbon ($\delta^{13}$C) and nitrogen ($\delta^{15}$N) has also been attempted.
(Hobson, 2006; Hupp et al., 2018). My interest was in exploring the possibility that, in addition to identifying plant types supporting dietary carbohydrates, stable isotopic measurements could similarly be used to trace where lipids were originally synthesized and stored to fuel migration.

Inferring origins of macronutrients isotopically requires the establishment of a clear and predictable relationship between synthesized tissue and environmental sources. The isotopic difference or discrimination (Δ) provides a means of linking tissue isotope values back to their source (Wassenaar, 2019) and is a fundamental challenge as several factors can presumably affect the discrimination process, including diet quality, metabolism, and metabolic routing (Podlesak & McWilliams, 2007). Isotopic fractionation, a term restricted to the specific isotopic change that takes place between a single chemical substrate and its product in a biogeochemical reaction, can be influenced by the nature and rate of such reactions (Wassenaar, 2019). Tissue synthesis typically involves multiple fractionation events making up an overall isotopic discrimination between diet and tissue (DeNiro & Epstein, 1977; Sessions et al., 1999; Hobson & Wassenaar, 2019). Lipid δ²H values can be affected by isotopic compositions of biosynthetic precursors, exchanges accompanying biosynthesis, and hydrogenation during biosynthesis (Sessions et al., 1999). When carbohydrates are ingested, they are primarily broken down into glucose (Droste & Zebe, 1974) in preparation for glycolysis. Glycolysis provides pyruvate, which is converted into acetyl-CoA (McCue, 2011; Shi & Tu 2015). This is the primary reactant for de novo lipid synthesis within the body of most organisms, including birds (Crabtree & Newsholme, 1975). Lipogenesis begins in the liver and adipocytes (Pearce, 1980; Zaefarian et al., 2019), and involves many enzymes and transporters (Klasing, 1998). With such complex biochemistry, it is not clear how well lipid isotope values can be consistently linked to the environments where they were produced.

The behavior of hydrogen isotopes in metabolic processes is poorly understood and is complicated by the fact that environmental hydrogen isotopes can exchange with hydrogen isotopes bound to oxygen and nitrogen. Only carbon-bound hydrogen does not readily exchange, meaning that once lipids are formed (involving primarily carbon-hydrogen bonds), they will no longer exchange with hydrogen isotopes present in body
water (Schimmelmann, 1991; Hobson & Wassenaar, 2019). However, in the case of precursor molecules in lipid synthesis, hydrogen is exchangeable with body water. For example, Hobson et al. (1999) found that drinking water $^2$H contributed about 18% of hydrogen to egg lipids in captive Japanese Quail (Coturnix japonica). Similarly, Wolf et al. (2011) found that for House Sparrows (Passer domesticus), drinking water $^2$H contributed greatly to a number of tissues (plasma, red blood cells, and feathers were 17±4%, 14±2%, and 18±3%, respectively).

In addition to sampling lipids in birds through biopsy (Clerc et al., 2017) or destructive sampling (i.e. sacrifice of individual), the isotopic examination of metabolic by-products can be considered. During a fed state, birds will primarily use dietary carbohydrates to fuel metabolic processes (McCue, 2011). During periods of fasting, birds will metabolize other stored tissues such as lipids (Pierce & McWilliams, 2005; McCue & Welch, 2016). Lipids are converted into acetyl-CoA using various enzymatic processes (Weber, 1992). Acetyl-CoA then enters the Krebs cycle to produce ATP, CO$_2$, and water (McCue, 2011). The CO$_2$ and water released from the birds during respiration would consist of then, carbon and hydrogen isotopes reflecting stored lipid sources of birds when fasted and the carbohydrate (dietary) sources of birds when fed (fuel use pattern seen in Smith et al., 2007). The isotopic measurement of breath for both isotopes is hence a viable means of tracing substrates used for metabolism (e.g. Hatch et al., 2002; Welch et al., 2016).

My objective was to investigate the feasibility of using $^{\delta^{13}}$C and $^{\delta^2}$H measurements of stored lipids in migratory birds to infer the geographic origins and/or the photosynthetic pathway of nutrients used to synthesize those fuel stores. I used captive White-throated Sparrows (Zonotrichia albicollis), a short-distance migratory passerine in North America (Falls & Kopachena, 2010) that is amenable to captive rearing and diet manipulations (Smith et al., 2007). Captured birds were fed isotopically distinct carbohydrate/oil ($^{\delta^{13}}$C, $^{\delta^2}$H) and water ($^{\delta^2}$H) diets to evaluate the potential for using the isotope approach to derive information on geographic origins of dietary nutrients. Linear calibration functions were generated between lipid and dietary source for both $^{\delta^2}$H and $^{\delta^{13}}$C using two points. Many calibration relationships have been determined to be linear in nature (e.g. Wolf et al., 2011; Hobson et al., 2012) thus, a two-point calibration function can be reasonably
encompass the discrimination relationship. Establishing this function can be considered the fundamental first step in deriving lipid-specific isoscapes for migratory organisms.

3.2 Methods

**Captive Trials:** Twenty-four adult White-throated Sparrows were housed at the Advanced Facility for Avian Research (AFAR), University of Western Ontario, Canada (all protocols were approved by the Canadian Council for Animal Care; protocol number: 2015-055). Birds had been used for another experiment, having been collected at the Long Point Bird Observatory (42.58°N, 80.40°W). They were weighed and placed on diet restrictions to reduce fat stores present prior to my experiment. Birds were originally fed 10g of Mazuri Small Bird Feed (St. Louis, United States of America) combined with small bird seed mix in a 1:1 ratio daily. During the diet reduction phase, birds were given 7 g of feed mix daily for 4 days and then 5 g for another 5 days. During this time birds were given tap water *ad libitum* and their mass was measured to 0.1g on an electronic balance (Accurat, 2000, Wedo, Dieburg Germany). Visual assessment of furcular lipid stores was preformed every 2 days. Dietary limitation was stopped when visible lipid stores were significantly reduced, and individuals had reached minimum weights of 20-23 g. During the reduction and treatment phases, birds were held at an average of 21°C with 12 hours of artificial light. Following the reduction phase, birds were separated into four groups, each with three females and three males. Each group was given five days to acclimate to a corresponding treatment diet under *ad libitum* (20 g of diet per bird a day) conditions prior to the official beginning of the trial period. Any birds that showed a decrease in mass during this adjustment period for three consecutive days were removed from the study. Each group of sparrows was fed a specific homogenized carbohydrate-based treatment diet (Table 3.1; based on Pierce & McWilliams, 2004).
Table 3.1 Composition of semi-synthetic diet fed to sparrows for 4 months
(composition for ~1000g).

<table>
<thead>
<tr>
<th>Component</th>
<th>Mass (g)</th>
<th>% Dry Mass</th>
<th>% Wet mass</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water (ml)</td>
<td>650 (ml)</td>
<td>--</td>
<td>68.6</td>
</tr>
<tr>
<td>Starch (Corn or Tapioca)</td>
<td>192</td>
<td>64.4</td>
<td>20.3</td>
</tr>
<tr>
<td>Casein*</td>
<td>41</td>
<td>13.8</td>
<td>4.3</td>
</tr>
<tr>
<td>Oil (corn or canola)</td>
<td>15</td>
<td>5.0</td>
<td>1.6</td>
</tr>
<tr>
<td>Vitamin Mixture**</td>
<td>6</td>
<td>2.0</td>
<td>0.6</td>
</tr>
<tr>
<td>Briggs N’Salt mixture**</td>
<td>18</td>
<td>6.0</td>
<td>1.9</td>
</tr>
<tr>
<td>Celufil***</td>
<td>10</td>
<td>3.4</td>
<td>1.0</td>
</tr>
<tr>
<td>Agar****</td>
<td>16</td>
<td>5.4</td>
<td>1.7</td>
</tr>
</tbody>
</table>

*Casein: Alfa Aesar by Thermo Fisher Scientific (#J12845-Q1)
**Vitamin Mixture: MP Biomedicals (#ICN90545401)
**Briggs N Salt Mixture: MP Biomedicals (#902834)
***Cellulose fibrous: Sigma-Aldrich (#C6288)
****Agar: G-Biosciences (#RC-004)

Each diet was composed of isotopically distinct oil/carbohydrate components (Table 3.2) and one of two drinking water sources using a 2x2 design: 1) high $^{13}$C (C4 corn starch and corn oil) with high $^2$H water, (2) high $^{13}$C (C4 corn starch and corn oil) with low (tap) $^2$H water, (3) low $^{13}$C (C3 tapioca starch and canola oil) with low (tap) $^2$H water, and a low $^{13}$C (C3 tapioca starch and canola oil) with high (spiked) $^2$H water.
Table 3.2 Average isotopic ($\delta^{13}$C and $\delta^{2}$H) values of diet and dietary components of synthetic diet. Sample size is 3 in all cases.

<table>
<thead>
<tr>
<th>Diet Component</th>
<th>$^{13}$C</th>
<th>$^{2}$H</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean ± SD (‰)</td>
<td>Mean ± SD (‰)</td>
</tr>
<tr>
<td>C3 Starch</td>
<td>-26.61 ± 0.03</td>
<td>-95.77 ± 0.83</td>
</tr>
<tr>
<td>C4 Starch</td>
<td>-11.15 ± 0.02</td>
<td>-20.99 ± 1.40</td>
</tr>
<tr>
<td>Casein</td>
<td>-26.69 ± 0.06</td>
<td>-116.27 ± 0.41</td>
</tr>
<tr>
<td>Vitamins</td>
<td>-12.67 ± 0.06</td>
<td>--</td>
</tr>
<tr>
<td>Agar</td>
<td>-19.37 ± 0.08</td>
<td>--</td>
</tr>
<tr>
<td>Briggs Salt Mixture</td>
<td>-15.04 ± 1.15</td>
<td>--</td>
</tr>
<tr>
<td>Celufil</td>
<td>-26.19 ± 0.01</td>
<td>--</td>
</tr>
<tr>
<td>C3 Diet</td>
<td>-26.18 ± 0.03</td>
<td>-99.56 ± 2.93</td>
</tr>
<tr>
<td>C4 Diet</td>
<td>-15.64 ± 0.07</td>
<td>-54.76 ± 3.58</td>
</tr>
<tr>
<td>C3 oil</td>
<td>-27.99 ± 0.07</td>
<td>-260.08 ± 1.39</td>
</tr>
<tr>
<td>C4 oil</td>
<td>-15.08 ± 0.03</td>
<td>-207.59 ± 0.51</td>
</tr>
<tr>
<td>Spike water</td>
<td>--</td>
<td>303.90 ± 8.93</td>
</tr>
<tr>
<td>Unspike water</td>
<td>--</td>
<td>-54.44 ± 2.45</td>
</tr>
</tbody>
</table>

After birds acclimated to their new diets, they were held on these diets for the duration of the study. I used quantitative magnetic resonance (QMR) (EchoMRI Whole Body Composition Analyzer version 140320, Houston, Texas) to estimate individual lean mass and fat mass at the beginning and end of synthetic diet feeding period (Taicher et al., 2003). The instrument was calibrated using 18g of canola oil (17.9g oil, 0g lean mass, 0g water). Birds were maintained on the treatment diet for four months and until visible furcicular fat levels had increased to extractable levels. At that time, breath CO$_2$ and breath H$_2$O isotopic analyses were performed on each individual (below). Birds were then sacrificed, and lipids extracted from subcutaneous depots at the furcular hollow. Carbon isotopic discrimination ($\Delta^{13}$C) is based on the isotopic ($\delta^{13}$C) difference between dietary starch and lipids, which can be depicted through a calibration function. Hydrogen isotopic discrimination ($\Delta^{2}$H) is more complex and is influenced by both H-exchange
occurring between sparrow body water and lipid precursors, as well as δ²H differences between dietary sources and sparrow lipids (Hobson et al., 1999; Sessions et al., 1999). Here, for water and starch sources, I focused on δ²H discrimination associated with starches. I reasoned 1) that from a mass balance perspective, many species of birds in preparation for migration will use carbohydrates as the primary reactant for lipid synthesis (Pierce & McWilliams, 2005) and consequently the isotopic contribution of hydrogen to lipids and 2) that sparrow body water would be closely linked to environmental water δ²H integrating into the food web through common carbohydrates sources such as seeds fruits and grains (see Discussion).

**Stable isotope measurements of breath CO₂ and H₂O:** In preparation for the fasted breath analysis, food and water were restricted from 9:30 pm until the next morning at 7:00 am (overnight fast as preformed in Smith et al., 2007). Following this, for CO₂ measurements, birds were individually sampled by placing their head into a customized breath chamber through a slit in a latex cover. The chamber received 2.5 l/min of CO₂-free air (Praxair, gas mixture). Birds were held in place and allowed to breathe normally for 30-45 seconds while the exhaled breath was diverted from the breath chamber into a Nafion exchange filter to remove breath water vapour. Filtered air was then passed at a rate of 1 l/min to a Los Gatos Research Isotopic CO₂ laser analyzer (CCIA - 38 - ER, Model 2-912, Mountain View, California) calibrated with CO₂ compressed gas (1977 ppm, -12.99 ‰, Praxair) (Figure 3.1A). Following this, the same bird was switched to a similar breathing chamber attached to a Los Gatos Research Isotopic Water Analyzer (IWA - 45 - EP, Model 912, Mountain View, California). Dry (water-free) compressed air flowed into the breath chamber at a rate of 1 l/min and the breath exhaled by the birds was diverted to the analyzer at a rate of 0.5 l/min (Figure 3.1B). Birds were held in the chamber for an average of 3 minutes to ensure a stable trace of δ²H. After the fasting experiments, all birds were returned to their cages and supplied with ad libitum synthetic diet and water for the rest of the day and night as normal. The following day, after the birds had time to feed on the diet in the morning, breath analysis for CO₂ and H₂O was performed as described above. Following this experiment, the birds were left to feed and rest normally in their cages until sacrifice.
Figure 3.1 CO₂ (A) and water vapour (B) breath isotopic analysis system set up for data collection.

At the end of feeding trials, birds were sacrificed using isoflurane followed by decapitation, and bodies were kept frozen (-20°C). Lipid was excised primarily from the furcular subcutaneous depot. Excised lipids were then purified with a 2:1 (v:v) chloroform:methanol solution for 48 hours and dried in a fume hood >> 24 hours (23°C, 101.3 kPa). Each bird lipid sample was weighed into tin (δ¹³C) or silver (δ²H) capsules and analyzed via CF-IRMS at the LSIS-AFAR laboratory at University of Western Ontario or at the Environment and Climate Change Canada stable isotope facility in Saskatoon, Saskatchewan. Using the same method as previously mentioned sub samples of pectoral muscle, liver and large intestine were also prepared and sampled for proteinaceous δ²H and δ¹³C analysis.

**Stable Isotope Measurements of lipids:** For δ²H analyses, 0.35mg of lipid were loaded into silver capsules and crushed. These samples were loaded into a uni-prep (Eurovector; Milan, Italy) carousel at room temperature, evacuated and kept under a pressurized He atmosphere. Samples were directly introduced into a Eurovector 3000 elemental analyser.
and combusted pyrolytically at 1350 °C on glassy carbon. Resultant gases were separated and introduced via a Conflo interface to a Thermo Delta V Plus stable isotope mass spectrometer (Thermo Instruments, Bremen, Germany). All tissue samples were calibrated relative to silver-encapsulated water USGS standards (VSMOW2: 0 ‰; USGS 46: -235.8 ‰). Within-run standards also included CBS (-197 ‰) and KHS (-54.1 ‰) keratin standards to adjust for instrument drift. Based on within-run replicates of standards, I estimated measurement error to be ± 3 ‰.

For δ¹³C measurements, approximately 0.35 ± 0.02 mg of samples were weighed into 4 x 3.2 mm tin pressed capsules. Concurrent δ¹³C and δ¹⁵N analyses were performed using a Costech Elemental Analyzer coupled to a Thermo Delta Plus XL isotope ratio mass spectrometer (Thermo Instruments, Bremen, Germany) operated in continuous flow mode, with helium carrier gas but only δ¹³C values are reported here. Two standards, USGS-40 and USGS-41, were included for every ten samples and two internal laboratory standards, powdered keratin (MP Biomedicals Inc., Cat No. 90211, Lot No.9966H) and IAEA-CH-6, were included to monitor instrument drift and provide a check on accuracy over the course of each analytical session. Values ofδ¹³C were calibrated to VPDB using USGS-40 (± 0.1 ‰ 1 SD, n = 8, accepted δ¹³C = -26.4 ‰) and USGS-41 (± 0.1 ‰, n = 8, accepted δ¹³C = +37.6 ‰). Measurement error was ± 0.1 ‰ for δ¹³C.

Drinking water δ²H analyses were performed using an Off Axis Integrated Cavity Output Spectroscopy (OA-ICOS) using a Los Gatos Research DLT-100 laser spectrometer (Mountain view California). Two calibrated reference waters (INV1 δ²H = -217.7 ‰ and ROD3 δ²H = -3.9 ‰, respectively) were used to normalize raw delta values to the VSMOW–SLAP scale. To minimize memory effects, samples and reference waters were injected 9 times and the last 5 measurements were averaged to obtain the final raw delta values. Precisions as determined by replicate analyses of samples and reference waters were ± 1 and 0.1 ‰.

**Statistical Analysis:** All statistical tests (Kruskal Wallis tests and GLMs) were performed using RStudio (version 3.4.2 (2017-09-28)) as required. Graphs were generated using Microsoft Excel 2010. GLMs were used for both body lipid data
comparisons and breath metabolite comparisons to diet as the data were not normally distributed. When comparing breath metabolites between fed and fasted states, Kruskal Wallis tests were used.

3.3 Results

Birds treated with spiked and unspiked water in their adult diet had correspondingly high and low lipid $\delta^2$H values (GLM, df= 3, 19 F= 65.79, $P<0.0001$) (Figure 3.2, Table 3.3) and birds treated with C3 or C4 carbohydrate sources also showed corresponding differences in their $\delta^{13}$C values of synthesized lipids (GLM, df= 3, 19, F= 2128.1, $P<0.0001$) (Figure 3.3, Table 3.3). Unspiked C4-treated birds had more positive lipid $\delta^2$H values compared to the unspiked C3 group (Kruskal test, $\chi^2 = 5$, df = 1, 8, $p = 0.02$) indicating differences in $\delta^2$H values of the carbohydrate fraction. There was no effect of sex on the $\delta^{13}$C or $\delta^2$H value of body lipid in the sparrows (Appendix B, Table B.1).

![Box plot of $\delta^2$H values for different diets](image)

Figure 3.2 Differences in $\delta^2$H values of sparrow body lipid in relation to differential diet treatments. (*) indicate statistically significant differences in the $\delta^2$H value of body lipid on that diet ($P<0.05$).
Figure 3.3 Differences in $\delta^{13}C$ values of sparrow body lipid in relation to differential diet treatments. (*) indicate statistically significant differences in the $\delta^{13}C$ value of body lipid on that diet (P<0.05).

Table 3.3 Average isotopic ($\delta^{13}C$ and $\delta^2H$) values of sparrow lipids under isotopically distinct diets. S refers to “spiked” water and U to “unspiked” (tap) water.

<table>
<thead>
<tr>
<th>Diet</th>
<th>Mean $\pm$ SD (‰)</th>
<th>N</th>
<th>Mean $\pm$ SD (‰)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$^{13}C$</td>
<td></td>
<td>$^2H$</td>
<td></td>
</tr>
<tr>
<td>C3/S</td>
<td>-29.37 ± 0.13</td>
<td>5</td>
<td>-191.29 ± 14.52</td>
<td>5</td>
</tr>
<tr>
<td>C3/U</td>
<td>-28.83 ± 0.64</td>
<td>4</td>
<td>-284.87 ± 6.97</td>
<td>4</td>
</tr>
<tr>
<td>C4/S</td>
<td>-16.62 ± 0.12</td>
<td>6</td>
<td>-188.15 ± 14.52</td>
<td>6</td>
</tr>
<tr>
<td>C4/U</td>
<td>-16.49 ± 0.39</td>
<td>5</td>
<td>-251.81 ± 5.54</td>
<td>5</td>
</tr>
</tbody>
</table>

Based on the (unspiked) tap water source, I derived a calibration function linking $\delta^2H$ in sparrow lipids ($\delta^2H_{lip}$) to diet (C3 and C4) carbohydrates ($\delta^2H_c$) as $\delta^2H_{lip} = 0.63 \times \delta^2H_c - 121.06‰$ (Figure 3.4).
Figure 3.4 $\delta^2$H value of body lipids of birds in relation to the $\delta^2$H value of dietary carbohydrate.

Similarly, the calibration function between carbohydrate source $\delta^{13}$C ($\delta^{13}$C$_c$) and synthesized body lipid $\delta^{13}$C ($\delta^{13}$C$_{lip}$) on a fixed tap water source was established; $\delta^{13}$C$_{lip}$ = 0.80* $\delta^{13}$C$_c$ – 7.59‰ (Figure 3.5).
Figure 3.5 δ¹³C value of body lipids of birds in relation to the δ¹³C value of dietary carbohydrate.

From this information a two-endpoint isotopic mixing model was used to determine the proportional contribution of each dietary carbohydrate source to the δ²H and δ¹³C lipid isotopic value of fed sparrows. In the δ¹³C of sparrow lipid, 80% (± 5%; Appendix B Equation B.1) of the total isotopic value came from the dietary starch and 20% came from the dietary oil. (± 5%; Appendix B Equation B.1). Using the unspiked treatment groups, I estimated that starch contributed 44 ± 3% of the sparrow δ²H lipid value and drinking water 36 ± 6% (Appendix B Equation B.2).

Breath water vapour δ²H of fed and fasted birds differed according to the tap/spike water source as expected (Figure 3.6, Table 3.4) (GLM, df = 3,19 F = 33.85, P < 0.0001; GLM, df = 3,19, F = 18.36, P < 0.0001 for fed and fasted respectively). When comparing the breath δ²H among the same diet treatment but different feeding states, fasted birds all had a more negative δ²H value compared to fed birds (Kruskal test: C3/S χ² = 6.81, df = 1,9, p = 0.009, C3/U χ² = 5.33, df = 1,7, p = 0.008, C4/S χ² = 8.31 df = 1,11, p = 0.003, C4/U χ² = 5.77, df = 1,9, p = 0.008).
Figure 3.6 δ²H value of breath water vapour of birds raised on fixed diets during fed and fasted states. S refers to “spiked” water and U to “unspiked” (tap) water. (*) indicates statistically significant differences δ²H value of breath water vapour in fed and fasted birds on same diet treatment (P<0.05).
Table 3.4 Average isotopic ($\delta^{13}C$ and $\delta^2H$) values of sparrow breath when fed or fasted under isotopically distinct diets. S refers to “spiked” water and U to “unspiked” (tap) water.

<table>
<thead>
<tr>
<th>Feeding State</th>
<th>Diet</th>
<th>$\delta^{13}C$ Mean ± SD (‰)</th>
<th>N</th>
<th>$\delta^{13}C$ Mean ± SD (‰)</th>
<th>N</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Fed</td>
<td>C3/S</td>
<td>-23.57 ± 2.28</td>
<td>5</td>
<td>-25.13 ± 24.77</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>C3/U</td>
<td>-24.54 ± 2.14</td>
<td>4</td>
<td>-108.05 ± 6.46</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>C4/S</td>
<td>-13.43 ± 1.93</td>
<td>6</td>
<td>-21.60 ± 22.05</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>C4/U</td>
<td>-13.00 ± 1.03</td>
<td>5</td>
<td>-104.16 ± 15.70</td>
<td>5</td>
</tr>
<tr>
<td>Fasted</td>
<td>C3/S</td>
<td>-19.84 ± 0.64</td>
<td>5</td>
<td>-94.974 ± 13.63</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>C3/U</td>
<td>-19.57 ± 0.73</td>
<td>4</td>
<td>-135.97 ± 5.62</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>C4/S</td>
<td>-8.09 ± 1.15</td>
<td>6</td>
<td>-94.62 ± 12.76</td>
<td>6</td>
</tr>
<tr>
<td></td>
<td>C4/U</td>
<td>-8.96 ± 1.28</td>
<td>5</td>
<td>-129.86 ± 10.31</td>
<td>5</td>
</tr>
</tbody>
</table>

Breath CO$_2$ $\delta^{13}C$ of fed and fasted birds differed according to the C3/C4 diet source as expected (Figure 3.7, Table 3.4) (GLM, df = 3,19, $F = 55.99$, $P < 0.0001$; GLM, df = 3,19, $F = 202.91$, $P < 0.0001$ for fed and fasted respectively). However, for any given diet treatment (C3 or C4), birds differed in their breath CO$_2$ $\delta^{13}C$ values where fasted birds had more positive breath $\delta^{13}C$ values compared to fed birds (Kruskal test: C3/S $\chi^2 = 4.81$, df = 1.9, $p = 0.02$, C3/U $\chi^2 = 5.33$, df = 1.7, $p = 0.03$, C4/S $\chi^2 = 8.31$, df = 1.11, $p = 0.003$, C4/U $\chi^2 = 6.81$, df = 1.9, $p = 0.009$).
Figure 3.7 \( \delta^{13}C \) value of breath CO\(_2\) of birds raised on fixed diets during fed and fasted states. S refers to “spiked” water and U to “unspiked” (tap) water. (*) indicates statistically significant differences \( \delta^{13}C \) value of breath CO\(_2\) in fed and fasted birds on same diet treatment \((P<0.05)\).

### 3.4 Discussion

I have demonstrated that synthesized lipids in adult White-throated Sparrows clearly reflect the stable isotope values of their diet. I demonstrated this for both \( \delta^{13}C \) and \( \delta^2H \) for the carbohydrate fraction of diet and for \( \delta^2H \) in drinking water. For both isotopes, lipids were isotopically more negative (i.e. more depleted in \( ^{13}C \) and \( ^2H \)) than the dietary and water sources from which they were formed (Table 3.2 & 3.3, previously discussed in DeNiro & Epstein, 1977; Sessions et al., 1999; Podlesak & McWilliams, 2007). Furthermore, isotopic signal differences present in the diet were traceable to stored lipids and in the CO\(_2\) and H\(_2\)O products of lipid metabolism in breath. These results indicate that stable isotope analyses of endogenous lipid reserves and their subsequent metabolic by-products can be assayed in migratory passerines to investigate both where lipids were synthesized and whether birds were foraging locally or depending on stored reserves for metabolism.
Lipid δ²H values are potentially useful for tracing provenance of lipid synthesis as plant tissue δ²H at the base of local foodwebs is derived from precipitation, which in turn shows strong long-term average continental patterns (Vander Zanden et al., 2016; Bowen & West, 2019). Pertaining to this experiment, water treatments were used to display the integration of body water into lipid. However, in the natural world the water and carbohydrate sources that constitute fruits/seeds/grains may be isotopically similar as they are synthesized from a common precipitation δ²H source (Farquhar et al., 2007; Cernusak et al., 2015). Frugivorous or granivorous birds are expected to synthesize lipids primarily from carbohydrates in fruits and seeds (Moermond & Denslow, 1985; Guglielmo, 2018). Isotopic values of δ⁶H in plant leaf water and cellulose components of plants have been shown to follow δ²H values in precipitation (Bowen & West 2019; Mora & Zanazzi, 2017) and I expect plant carbohydrate sources that constitute seeds and fruits to follow a similar pattern. In this experiment, discrimination was explored using the δ²H values of the carbohydrate source as a proxy for both drinking water and carbohydrates in sparrow diets in the natural environment. I provide, for the first time, a calibration function linking plant-based carbohydrate δ²H values with those of synthesized lipids. Providing the δ²H value in lipids reflects the δ²H value of dietary carbohydrate and drinking water δ²H is closely linked to plant carbohydrate δ²H regionally, I therefore expect the calibration function that I have derived to be similar to that which will apply to wild birds. My study provides strong evidence that sparrow lipids correspond with body water δ²H, carbohydrate δ²H, and oil δ²H. This supports my hypothesis that lipids can ultimately be used to determine the origins of lipids stored by migratory organism. Thus, it should be feasible to trace the origin of lipids geographically in the same way that δ²H measurements in keratins and other animal tissues are used (e.g. Bairlein et al., 2016).

With the assumption that fruits and seeds should have isotopic values that are reflective of the plant source and the geographic environment, there should be a strong isotopic relationship between environmental waters and synthesized lipids geographically. If drinking water and plant carbohydrate δ²H values are not both correlated with the environmental isotopic values of precipitation δ²H, then the calibration becomes more complex. Hydrogen isotopes in body water can also integrate directly in the carbohydrate
breakdown and lipid synthesis process. For example, the breakdown of starch by amylases (Pollock, 2006) and the extension of lipids by thioesterases (Klasing, 1998) both incorporate water and, in the case of experimental birds, body water is largely derived from drinking water (Sessions et al., 1999; Wolf et al., 2011). That may be less so in the field where wild birds gain body water also from diet. Indirectly, when molecules are being broken down and rebuilt to produce lipids, hydrogen isotopes can readily exchange with the environment in the precursor stages due to hydrogen-oxygen bonds being less stable than other common biochemical hydrogen bonds such as hydrogen-carbon (Myint et al., 2014; Soto et al., 2017). Particular to this experiment, the spike of the water treatment used was extreme (i.e. $\pm 303.9 \text{‰}$) and may have influenced the physiology of the birds (Katz et al., 1957; Xie & Zubarev, 2014). Considering the previous literature (e.g. Hobson et al., 1999; Wolf et al., 2011) and the proportional contribution of drinking water established in this experiment, it would be beneficial to explore the impact of drinking water on the isotopic value of various tissues using more conservative (i.e. $\sim +100 \text{‰}$) spiked water treatments.

My isotopic analysis of sparrow breath indicated clearly that differences of both $\delta^{13}\text{C}$ in breath CO$_2$ and $\delta^2\text{H}$ in breath water vapour reflected those of dietary substrates and drinking water. In the fed state, both isotopes behaved as predicted, indicating that the dietary sources that were being readily provided were used as metabolic fuel (Table 3.2 & 3.4; McCue, 2011; McCue & Welch, 2016). Within the fasted and fed trials, breath water vapour $\delta^2\text{H}$ values reflected differences in isotopic sourcing of drinking water. I interpret the more negative $\delta^2\text{H}$ value of breath water vapour from fasted individuals as reflecting the use of stored lipids as fuel when carbohydrates were unavailable (McCue & Welch, 2016), as lipid $\delta^2\text{H}$ values tend to be more negative than the other macromolecules (Sessions et al., 1999). Therefore, lipid use and the dietary differences in lipid synthesis sources was reflected at the metabolic by-product level as well as directly in body lipid.

Results from the isotopic analysis of breath CO$_2$ for the fed and fasted trials were less clear. In the fed trials, breath CO$_2$ $\delta^{13}\text{C}$ values, as expected, followed the $\delta^{13}\text{C}$ values of the bulk dietary sources (Tables 3.2 and 3.4) as they were burned for fuel (Smith et al.,
2007; McCue, 2011). However, the fasted trials produced CO$_2$ $\delta^{13}$C values that were enriched in $^{13}$C compared to those of the fed trials and so were not consistent with the mobilization of stored lipids (as seen in Hayes, 2001; Engel et al., 2009) as I found with the trials involving $\delta^2$H in breath water vapour. My $\delta^2$H data were consistent with the concept of fasted birds using stored lipids for metabolism, but my $\delta^{13}$C data were not.

During periods of fasting, while lipid mobilization is common, it is not the only mode by which metabolism is fuelled (McCue, 2011; McCue et al., 2010; McWilliams et al., 2004). Many other sources of fuel, such as muscle glycogen, muscle tissue, and digestive organs, can also become metabolized (Piersma & Jukema, 1990; Pierce & McWilliams, 2004; McCue et al., 2010; Guglielmo, 2018) and thus the metabolic by-products in the breath could be the result of a combination of sources, as suggested by my breath CO$_2$ $\delta^{13}$C values. The breakdown of proteins will produce acetyl-CoA as well, such that breath CO$_2$ may have resulted from metabolism from proteinaceous tissues (McCue et al., 2010; Shi & Tu, 2015). To explore this, liver, large intestine, and muscle tissue samples were analyzed isotopically as potential fuel sources positive enough to account for the breath CO$_2$ $\delta^{13}$C values seen in the fasted sparrows. The sampled tissues did not account for the $^{13}$C enrichment seen in fasted breath CO$_2$ (Appendix B, Table B.2) but are likely also being used as fuel along with lipids.

The discrimination between the $\delta^{13}$C of fasted breath CO$_2$ and the $\delta^{13}$C of dietary oils was relatively similar for C3 and C4 treatments (Table 3.2 & 3.4, $\Delta \approx +8.9\%$). Dietary oil contributed about 20% of the body lipid and thus the similar discrimination seen here might be indicative of preferential metabolism of dietary oil stored in lipids rather than de novo synthesized lipids from carbohydrates during fasting. Both dietary oils provided were unsaturated (Nicolosi, 1997). Unsaturated fatty acids have been shown to be preferentially used as fuel in other avian studies (Price et al., 2008; Guglielmo, 2018). Although this does not explain the enrichment seen in breath CO$_2$ of fasted birds, it is reflective of the information that breath isotopic analysis can provide in determining dietary sources and fuel metabolism. When mammals are not provided with dietary resources and lipolysis is taking place, triacylglycerides are broken down into fatty acid chains and glycerol moieties (Weber, 1992; Subramaniam et al., 2011). Though most of
the fatty acids are broken down through oxidation (Weber, 1992), a substantial proportion of fatty acids can be recycled through re-esterification (Vallerand et al., 1999; Viscarra & Ortiz, 2013) (Figure 3.8).

![Biochemical Pathway of Lipolysis](image)

**Figure 3.8 Simplified biochemical pathway of lipolysis from adipocytes in birds. Re-esterification can be a possible site of $^{12}$C recycling causing more enriched lipids to be oxidized initially.**

Re-esterification has been explored in a study conducted on fuel use patterns on Ruffs (*Philomachus pugnax*). Almost 50% of the fatty acids were recycled through re-esterification during periods of high stress (Vaillancourt & Weber, 2007). If the re-esterification biochemical pathway discriminates against $^{13}$C, as seen commonly for other enzymes associated with lipids (DeNiro & Epstein, 1977), the fatty acid that is transferred into the oxidation pathway (instead of re-esterification) would be more enriched in $^{13}$C. If more enriched lipids are being used as fuel, the enrichment of $^{13}$C in breath CO$_2$ seen in this experiment is plausible. The QMR analysis done on the sparrows indicates that body lipid was present in the sparrows at the time of fasting as a potential fuel source (Appendix B, Table B.3). Water vapour $\delta^2$H values also indicate lipid fuel use. Isotopic behavior of hydrogen and carbon isotopes are clearly not always correlated, and differential enzymatic selection or differential source processing pathways were likely involved for each isotope during metabolism (DeNiro & Epstein, 1977; Sessions et al., 1999). However, no definitive conclusions can be drawn without further exploration of the biochemical causes behind this enrichment.

Lipids can be a very useful source of information from an isotopic perspective. Lipids are stored, for purposes such as reproduction (Davis et al., 2016), and turned over for fuel. Lipids can provide additional information in isotopic studies pertaining to allocation and
origins of nutrients. In this study, isotopic discrimination between carbohydrate and lipid for both $\delta^{13}C$ and $\delta^2H$ was established. I assume that the calibration functions established here can be applied to other songbirds, especially those that have diets common to that provided to the sparrows (Caut et al., 2009). Such calibration functions can hypothetically be applied to environmental isoscapes to generate isoscapes that are lipid-specific or metabolite-specific that can be used to trace key regions for lipid synthesis and fuelling. Future studies should focus on refining isotopic calibrations linking avian lipids to $\delta^2H$ and $\delta^{13}C$ isoscapes to generate “liposcape” for this purpose.

Though laboratory experiments can provide useful information, experimental dietary treatments cannot account for the natural variations in drinking water and carbohydrate sources found in the natural environment (Dansgaard, 1964; O’Leary et al., 1992). A collection of passerine lipid samples, precipitation samples, and dietary plant sources across a latitudinal gradient for the purposes of isotopic analysis can lead to the generation of a calibration function much more reflective of true isotopic relationship between lipids and the environment. This sort of gradient sampling to establish calibration functions has been quite successful in other studies. Such a study was conducted to determine Neotropical migrant songbird breeding origins on wintering grounds (Hobson & Wassenaar, 1996). The $\delta^2H$ values of feathers collected from 14 different sites across North America were used generate a calibration function between $\delta^2H$ of feathers and $\delta^2H$ of seasonal precipitation (Hobson & Wassenaar, 1996). The tissue-specific isoscape generated using this function was then used to infer the breeding ground origin of songbirds sampled in Guatemala (Hobson & Wassenaar, 1996). A gradient-based study of lipid isotopes in passerines will embody all sources of discrimination from plants to lipids in the case of $\delta^{13}C$ and especially between environmental waters (GNIP) to lipids in the case of $\delta^2H$. 
3.5 References


Chapter 4

4 Conclusion

4.1 Introduction

The use of stable isotope measurements for tracking migration and tracing nutrient allocation patterns has focused mainly on metabolically inactive protein-based (keratin) and carbohydrate-based (chitin) structures (Hobson & Wassenaar, 2019), that provide information from fixed points in time during synthesis. Lipids in migratory organisms can be used as a fuel and can also be conserved from larval stages of insects (Cookman et al., 1984; Weber, 2009; Guglielmo, 2018). Lipids are also allocated to reproduction in mixed/capital strategy breeders, and preferentially metabolized depending on the conditions experienced by the migratory organism at present or during previous life stages (Price et al., 2008; Dhurmeea et al., 2018). Consequently, lipids are a source of isotopic information that reflects both synthesized and routed components, although they have thus far been relatively underrepresented in isotopic studies of migration and nutrient allocation.

My thesis explored the traceability of origins of bulk lipids using δ^{13}C and δ^{2}H measurements in birds and insects by establishing consistent isotopic relationships between diet and de novo synthesized lipids. Calibration functions between dietary carbohydrate isotopic values and de novo synthesized lipids were derived for both ^{13}C and ^{2}H in the true armyworm moth and the White-throated Sparrow. The proportional contributions of different isotopic sources to body lipid were also distinguished for ^{13}C and ^{2}H in both model organisms. Lipid isotopic values are not limited to providing information on stored lipid. As my thesis work showed, lipid δ^{2}H and δ^{13}C values during uptake in moths and catabolism in sparrows also provided information on dietary sources of lipids. Therefore, δ^{13}C and δ^{2}H values can be used to trace lipid origins, lipid metabolic routing during fuelling and catabolism, as well contributions of multiple pools of isotopic sources to body lipids.
4.2 Important Findings

4.2.1 Carbon
Calibration functions between body lipid and dietary carbohydrate for the moth and sparrow had similar slopes \((m \approx 0.8)\), which indicated comparable isotopic discrimination of carbohydrate \(\delta^{13}C\) values and \textit{de novo} synthesized lipid \(\delta^{13}C\) values between these species. I also found that the proportion of the total \(\delta^{13}C\) value of body lipid originating from the carbohydrate dietary source was similar for both model organisms (~80% from carbohydrate sources in both organisms). Larval lipid and dietary oil also contributed to the \(\delta^{13}C\) value of moth and sparrow body lipid, respectively. The differences between carbohydrate source \(\delta^{13}C\) values were also reflected in the change in body lipid \(\delta^{13}C\) value during the C4 to C3 nectar switch in the moths. Fed and fasted sparrow breath CO\(_2\) \(\delta^{13}C\) values also reflected C4 and C3 carbohydrate \(\delta^{13}C\) value differences. Regardless of species, lipid synthesis is expected to follow similar biochemical pathways (Crabtree & Newsholme, 1975) and this was supported by the similar calibration functions, as well as the C4 and C3 isotopic differences in experimental tissues, and proportional contribution of carbohydrates found between these two species.

4.2.2 Hydrogen
Both armyworms and sparrows varied in the slopes of the calibration function between \(\delta^2H\) of body lipid and \(\delta^2H\) of dietary carbohydrate source \((m \approx 1.3\) and 0.6 respectively\). This was consistent with there being more sources of variance in the hydrogen pathways to lipid synthesis compared to carbon pathways for both organisms (Sessions \textit{et al.}, 1999). There were also the influences of larval source lipid that was carried over into the adult stages of the moth that would not be present in the sparrow body lipid composition which can also cause the calibrations to vary between the two species. The contribution of carbohydrates to the \(\delta^2H\) value of total body lipid differed between moths and sparrows, reflecting a greater variety in the sources of \(\delta^2H\) and metabolic routing. Variations in metabolic routing can be driven by factors such as hydrogenation and isotopic exchange (Sessions \textit{et al.}, 1999) that would not affect \(^{13}C\) routing and also contribute to the differences in the calibration functions generated. The calibration
functions generated for hydrogen in this experiment were based on the assumption that the $\delta^2H$ values of lipid are primarily derived from the carbohydrates and that the $\delta^2H$ values of carbohydrates should be determined by the $\delta^2H$ values of precipitation (Farquhar et al., 2007; Cernusak et al., 2015). However, many studies have shown that metabolic water and drinking water (DeNiro & Epstein, 1981; Hobson et al., 1999a; Ehleringer et al., 2008; Wolf et al., 2011; Vander Zanden et al., 2016) can influence the $^2H$ value of organic tissues.

The $\delta^2H$ value of body lipid varied among treatment groups according to spiked or unspiked water treatments. This reflected the incorporation of drinking/nectar water sources into de novo synthesized lipids. The differences between water source $\delta^2H$ values were also reflected in the change in body lipid $\delta^2H$ value during the unspiked to spiked nectar switch in the moths. The differences between drinking water source $\delta^2H$ values were seen in the breath water vapor $\delta^2H$ values of both fed and fasted sparrows. However, the majority of $\delta^2H$ is commonly derived from dietary sources rather than derived directly from environmental water (Vander Zanden et al., 2016). In agreement with this fact, a relatively lower proportion of the $\delta^2H$ isotopic value (when compared to contribution of H from carbohydrates) of body lipid in both moths and sparrows was generated from nectar water and drinking water $\delta^2H$ respectively, though proportions varied with the use of spiked or unspiked treatment water sources in the sparrows. The discrimination of $\delta^2H$ values derived between drinking/nectar water and lipid and that derived between drinking water and breath water vapor as a whole should also be taken into consideration in future studies (See future studies).

### 4.3 Caveats and assumptions

Water sources that are unnaturally enriched or depleted in $^2H$ can alter the physiology and isotopic response of an organism to $^2H$ in ways that are unexpected (Katz et al., 1957; Cong et al., 2010). I used a high concentration of $^2H$-spiked water to make up nectar and drinking water. The strong influence of the $\delta^2H$ value of the water source incorporating into tissues on isotopic discrimination between environmental water and bulk organismal tissue was found in a study by Peters et al. (2012). That study was performed on cabbage looper caterpillars (Trichoplusia ni) raised on cabbage (Brassica oleracea) plants grown
on four treatments of $^2$H-enriched water. The calibration function between water source and caterpillar indicated that isotopic discrimination depended on the $\delta^2$H value of the water source even though the $\delta^2$H values of the source waters fell within the $\delta^2$H values typically found in precipitation in North America (Peters et al., 2012). My use of highly $^2$H-enriched water whose $\delta^2$H values were high compared to those naturally found in precipitation isoscapes (typically between -160‰ and +20‰) (Bowen, 2010; Terzer, et al., 2013) may have physiological and isotopic effects (Katz et al., 1957; Cong et al., 2010), and thus may not generate calibration functions that could be applied in the natural world.

One factor that was not taken into consideration was the effect of the temperature at which the moths were frozen for sacrifice (approximately -21°C). Freezing temperature for sacrifice can have an effect on the use of stored reserves (May, 1979) during the last stages of life prior to lipid extraction and isotopic analysis. During the time it takes for the moth to freeze (approximately 10-20 minutes), fuel could have been burned in an attempt to maintain body temperature. This could occur through muscle contractions (i.e. shivering) (Heinrich, 1987) or hemolymph circulation through the rapid oscillations of the ventral diaphragm, as both can be used for thermoregulation (Koladich et al., 2002). To reduce the potential effect of fuel use during sacrifice in future research, sacrificing methods should ensure a rapid death without the potential for rapid fuel use (i.e. liquid nitrogen freezing).

Analysis of breath $\delta^{13}$C and $\delta^2$H values in fed sparrows reflected readily metabolized dietary sources. Differences in $\delta^{13}$C values in the breath of fed and fasted sparrows may have indicated sources of fuel other than de novo synthesized lipids (McCue et al., 2010). Differences in fasted sparrow breath CO$_2$ $\delta^{13}$C values can also be a result of post-storage metabolic processing of lipids by the liver and adipocytes during mobilization of lipid and preferential use of different fatty acids for fuel (Vaillancourt & Weber, 2007; Guglielmo, 2018). Along with C3 and C4 carbohydrate sources, birds were also provided with a small amount of C3 or C4 oil. Both the oil and the carbohydrate contributed to the body lipid signature. However, fasted sparrow breath CO$_2$ $\delta^{13}$C values corresponded more with those of the dietary oils, indicating possible preferential use of dietary lipids.
over *de novo* synthesized lipids. A simple dietary experiment using one oil of a consistent isotopic value among treatment diets with isotopically distinct carbohydrate sources could be used to determine if the preferential use of lipids obtained directly from the diet is indeed an energetic process that was occurring in fasted sparrows. Another simple dietary experiment to test preferential dietary oil metabolism could use diet treatments with consistent carbohydrate sources and isotopically distinct dietary oils.

### 4.4 Future Studies

In future research, an isotopic discrimination experiment could be performed that uses multiple water sources with different δ²H values and a consistent isotopic carbohydrate source to establish if a consistent calibration function can be generated between environmental water sources and *de novo* synthesized lipids. For example, natural precipitation δ²H values only vary in a range of 200‰ in global δ²H isoscapes (Bowen, 2010; Terzer, *et al*., 2013) and thus diet treatments with fixed carbohydrate sources and drinking water treatments of +50‰, -50‰, and -150‰ could be used in a future study to generate drinking water-based calibration functions for δ²H. The calibration generated from that experiment can be used in mixing models in addition to the carbohydrate-based δ²H ones determined in my thesis. However, restricting the generation of calibration functions between lipids and dietary resources experimentally will lead to calibration functions that will not account for the natural variations and seasonal changes that occur at the level of the environmental source (Manabe & Holloway, 1975; Marryanna *et al*., 2017). Isotopic differences between plant components, such as cellulose and leaves, and precipitation δ²H values have been explored (Bowen & West 2019; Mora & Zanazzi, 2017). The isotopic relationships between precipitation δ²H values and nectar or fruit/seed δ²H values have not been investigated. The differences in isotopic discrimination between precipitation and nectars and fruits/seeds could be generated through latitudinal gradient sampling of δ²H values in wild nectars and fruits/seeds. Transect sampling of wild environmental sources and individual organisms has been readily used across various taxa to generate tissue specific isoscapes (Hobson & Wassenaar, 1996; Hobson *et al*., 1999b; Hobson *et al*., 2012; Bowen & West 2019). The precipitation-to-nectar/fruit discrimination in δ²H values could be used in conjunction
with the isotopic discrimination between nectar/fruit and body lipids established in my study to derive total discrimination between environmental waters and \textit{de novo} synthesized lipids. This relationship, in turn, could then be used to derive continent-wide lipid $\delta^2\text{H}$ isoscapes for use in assigning lipids to origins that can account for more of the natural variations seen in the environment (Bowen & West, 2019).

Lipids are composed of essential and non-essential fatty acids (Blem, 1990; Spector, 2000) and recent advances in isotopic techniques have made it possible to trace fatty acids isotopically (Bec \textit{et al}., 2011). The compound-specific isotopic approach can be used to refine current isoscapes, identify metabolic differences that can cause bulk isotopic tissue variations, and examine detailed movement and resource utilization across different environments (McMahon & Newsome, 2019; Whiteman \textit{et al}., 2019). Compound-specific $\delta^{15}\text{N}$ analysis of amino acids has revealed information on the source of diets and can be used to derive trophic positions of consumers (past and present) (McMahon & McCarthy, 2016; Dale \textit{et al}., 2011). Considering that organisms migrate using a diverse array of life history strategies, compound-specific isotopic analysis of essential and non-essential macronutrients, such as amino acids, have been used as a powerful tool to study the physiology of migrating animals that cannot be recaptured multiple times to provide information on spatial or temporal changes (McMahon \textit{et al}., 2011; Fogel \textit{et al}., 2016; Whiteman \textit{et al}., 2019).

In a similar manner to amino acids, essential and non-essential fatty acids are either integrated directly from the environment or generated from other sources (Pierce & McWilliams, 2005; Podlesak & McWilliams, 2007; Guglielmo \textit{et al}., 2017). Thus, the compound-specific analysis of fatty acids using $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values could be used to explore nutrient allocation, origins, and fuel use changes in organisms as specific fatty acids should have distinct $\delta^{13}\text{C}$ and $\delta^2\text{H}$ values based on source of synthesis. (Bec, \textit{et al}., 2011; Budge \textit{et al}., 2011; Whiteman \textit{et al}., 2019). This has been seen in a study on hawkmoths (\textit{Manduca sexta}) by Levin \textit{et al}.. (2017) where $^{13}\text{C}$-labelled palmitic acid and amino acids were fed to adult moths to determine nutrient allocation between reproduction and fuel use. The conservation of $^{13}\text{C}$-label isotopic signal at the specific amino acid and fatty acid scale showed that adult diet non-essential amino acids and
essential amino acids were used for fuel and reproduction while adult diet fatty acids (palmitic acid) were used exclusively as metabolic fuel. A recent example of the compound-specific isotopic approach of fatty acid analysis was a study conducted by Carter et al. (2019) that explored the rate of specific fatty acid turnover at rest and during exercise in Zebra Finches (*Taeniopygia guttata*) during a C4 to C3 diet shift. The $\delta^{13}C$ values of specific fatty acids in the flight muscles of the finches indicated a rapid turnover of both linoleic and palmitic acid (Carter et al., 2019). Long-chain polyunsaturated fatty acids had the slowest turnover, suggesting their selective retention during metabolism (Carter et al., 2019). Research on the compound-specific isotopic analysis of fatty acids using $\delta^2H$ is a newly developing field of research (McMahon & Newsome, 2019; Whiteman et al., 2019). However, $\delta^2H$ values of specific fatty acids found in sediment cores have been used as proxies for climatic and environmental change (Huang et al., 2004; Hou et al., 2006), indicating that specific fatty acids can be used to identify environments of fatty acid origin. In one study, compound-specific analysis of $\delta^2H$ values of n-alkane hydrocarbons from western honeybee (*Apis mellifera*) beeswax found a correlation between the $\delta^2H$ values of n-alkane hydrocarbons and both mean annual precipitation and tap water $\delta^2H$ values found at hive location (Tipple et al., 2012). Considering the growing focus on compound-specific approaches to isotopic analysis, I suggest that fatty acid $\delta^{13}C$ and $\delta^2H$ analyses can benefit the exploration of the origins, allocation patterns, and metabolic use of lipids in migratory organisms.

### 4.5 Concluding Remarks

The purpose of my thesis was to explore the use of stable-isotope measurements of lipids as a viable tracking tool to infer nutritional sources and metabolic routing of lipids in migrating birds and insects. Overall, this work indicates that lipid, whether being sampled when stored in the body or during metabolic use for fuel, is a tissue that consistently conserves isotopic information from its source. Predictable discrimination and conservation of source isotopic signatures is the primary requirement of using any tissue to trace migration by stable isotopes (Bowen et al., 2005; Caut et al., 2009; Bowen & West, 2019). My thesis confirmed that lipid isotopic values are traceable from multiple sources differing in time of incorporation and state of metabolic routing. With this
advancement and the fact that lipids are both stored and turned over during migration as fuel (Weber, 2009), sampling lipids for δ^{13}C and δ^{2}H in a migrating organism can be used to infer multiple facets of migratory life history such as recent refuelling sites, origins, and fuel use. Fundamentally, this research showed that lipids should be utilized alongside other tissues such as muscle, blood or keratin as a tool to trace migratory origins, stopover sites, and nutrient allocation patterns in a variety of migratory species.
4.6 References


Appendices

Appendix A: Chapter 2 Supplementary Material

Equation A.1 Proportion contribution of $^{13}$C from dietary sources and larval lipid to moth body lipid

1. Carbon contribution from diet (p) (C3-C4 change (unspike)): $\Delta \delta^{13}$C in lipid/$\Delta \delta^{13}$C in diet = 0.81 ± 0.11
2. Carbon contribution from larval diet (P): 1- carbon contribution from diet = 0.19 ± 0.11

Equation A.2 Proportion contribution of $^2$H from dietary sources and larval lipid to moth body lipid

1. Deuterium contribution from carbohydrate (p) (C3-C4 change (unspike)): $\Delta \delta^{2}$H in lipid/$\Delta \delta^{2}$H in carbohydrate = 0.68 ± 1.01
2. Deuterium contribution from water (p*) in C3 treatments (Unspike to Spike change): $\Delta \delta^{2}$H in lipid/$\Delta \delta^{2}$H in water = 0.13 ± 0.45
3. Deuterium contribution from water (P) in C4 treatments (Unspike to Spike change): $\Delta \delta^{2}$H in lipid/$\Delta \delta^{2}$H in water = 0.13 ± 0.43
4. Larval lipid contribution (F) in C3 treatments: $1 - p - p^* = 0.19 ± 1.11$
5. Larval lipid contribution (f) in C4 treatments: $1 - p - P = 0.19 ± 1.10$
6. Average larval lipid contribution (F*) in all treatments = 0.19 ± 1.56
Table A.1 Secondary statistical analyses for average isotopic ($\delta^{13}C$ and $\delta^2H$) values of moth lipids from both experiments

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Test</th>
<th>Treatment comparison</th>
<th>Df</th>
<th>$\chi^2$</th>
<th>T</th>
<th>P</th>
<th>df</th>
<th>$\chi^2$</th>
<th>T</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotopic discrimination</td>
<td>GLM</td>
<td>None-C3/U</td>
<td>4.81</td>
<td>2.14</td>
<td>0.06</td>
<td></td>
<td>4.81</td>
<td>-4.37</td>
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<td>&lt;0.01</td>
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<tr>
<td></td>
<td>GLM</td>
<td>Sex</td>
<td>4.81</td>
<td>0.33</td>
<td>0.74</td>
<td>4.81</td>
<td>-2.04</td>
<td>--</td>
<td>0.06</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Kruskal</td>
<td>C3/U-C4/U</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>1.34</td>
<td>--</td>
<td>11.89</td>
<td>0.00</td>
<td>0.05</td>
</tr>
<tr>
<td>Isotopic uptake</td>
<td>GLM</td>
<td>Day 2-Day 3</td>
<td>3.38</td>
<td>1.31</td>
<td>0.20</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
<td>--</td>
</tr>
<tr>
<td></td>
<td>GLM</td>
<td>Day 2-Day 4</td>
<td>3.38</td>
<td>-1.43</td>
<td>0.16</td>
<td>--</td>
<td>--</td>
<td>--</td>
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</tr>
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</table>

Appendix B: Chapter 3 Supplementary Material

Equation B.1 Proportion contribution of $^{13}C$ from dietary sources to sparrow body lipid

1. Carbon contribution from carbohydrate in diet (p) (C3-C4 change (unspike)): $\Delta\delta^{13}C$ in lipid/$\Delta\delta^{13}C$ in diet = 0.80 ± 0.05
2. Carbon contribution from oil in diet (P): 1- carbon contribution from starch = 0.20 ± 0.05

Equation B.2 Proportion contribution of $^2H$ from dietary sources to sparrow body lipid

1. Deuterium contribution from carbohydrate (p) (C3-C4 change (unspike)): $\Delta\delta^2H$ in lipid/$\Delta\delta^2H$ in carbohydrate = 0.44 ± 0.03
2. Deuterium contribution from carbohydrate (P) (C3-C4 change (spike)): $\Delta\delta^2H$ in lipid/$\Delta\delta^2H$ in carbohydrate = 0.04 ± 0.27
3. Assume dietary oil contribution (F) is as with carbon (as oil does not fractionate and thus proportions should be the same): 0.20 ± 0.05
4. Deuterium contribution from water (p*) in unspiked treatments: 1 - F – p = 0.36 ± 0.06

5. Deuterium contribution from water (P*) in spiked treatments: 1 - F – P = 0.76 ± 0.28

Table B.1 Secondary statistical analyses for average isotopic (δ¹³C and δ²H) values of sparrow lipids from the isotopic discrimination experiment

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Test</th>
<th>Treatment comparison</th>
<th>Df</th>
<th>χ²</th>
<th>T</th>
<th>P</th>
<th>df</th>
<th>T</th>
<th>χ²</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Isotopic discrimination</td>
<td>GLM</td>
<td>Sex</td>
<td>3,19</td>
<td>--</td>
<td>-2.13</td>
<td>0.06</td>
<td>3,19</td>
<td>0.63</td>
<td>--</td>
<td>0.54</td>
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</table>

Table B.2 Average isotopic (δ¹³C) values of protein-based sparrow tissue samples under isotopically distinct diets. S refers to “spiked” water and U to “unspiked” (tap) water. Sample size is 2 in all cases.

<table>
<thead>
<tr>
<th>Diet Treatment</th>
<th>Liver Mean ± SD (‰)</th>
<th>Muscle Mean ± SD (‰)</th>
<th>Large Intestine Mean ± SD (‰)</th>
</tr>
</thead>
<tbody>
<tr>
<td>C3/S</td>
<td>-25.58 ± 0.007</td>
<td>-25.54 ± 0.007</td>
<td>-25.33 ± 0.05</td>
</tr>
<tr>
<td>C3/U</td>
<td>-25.60 ± 0.11</td>
<td>-25.34 ± 0.25</td>
<td>-25.45 ± 0.34</td>
</tr>
<tr>
<td>C4/S</td>
<td>-21.64 ± 0.29</td>
<td>-22.23 ± 0.23</td>
<td>-20.49 ± 0.39</td>
</tr>
<tr>
<td>C4/U</td>
<td>-21.69 ± 0.16</td>
<td>-22.27 ± 0.02</td>
<td>-21.07 ± 0.47</td>
</tr>
</tbody>
</table>
Table B.3 Sparrow QMR analysis of total mean lipid mass pre (after mass reduction period) and post experimental treatments. S refers to “spiked” water and U to “unspiked” (tap) water. Sample was run twice for each sparrow

<table>
<thead>
<tr>
<th>Band ID</th>
<th>Diet</th>
<th>Mass of lipid prior to experiment diet ± SD (g)</th>
<th>Mass of lipid at end of experiment diet ± SD (g)</th>
</tr>
</thead>
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<tr>
<td>86508</td>
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<td>2.56 ± 0.05</td>
<td>12.73 ± 0.26</td>
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<tr>
<td>86510</td>
<td>C4/U</td>
<td>1.08 ± 0.04</td>
<td>4.83 ± 0.04</td>
</tr>
<tr>
<td>86512</td>
<td>C4/U</td>
<td>1.83 ± 0.09</td>
<td>6.73 ± 0.16</td>
</tr>
<tr>
<td>86515</td>
<td>C4/S</td>
<td>1.90 ± 0.06</td>
<td>6.24 ± 0.07</td>
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<td>86516</td>
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<td>4.46 ± 0.13</td>
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<td>86518</td>
<td>C3/U</td>
<td>0.74 ± 0.01</td>
<td>1.46 ± 0.06</td>
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<tr>
<td>86519</td>
<td>C4/U</td>
<td>0.86 ± 0.03</td>
<td>7.88 ± 0.08</td>
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<tr>
<td>86523</td>
<td>C4/S</td>
<td>1.50 ± 0.03</td>
<td>5.90 ± 0.03</td>
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<td>86526</td>
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<td>2.62 ± 0.08</td>
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<td>86602</td>
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<td>86690</td>
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<td>88163</td>
<td>C4/U</td>
<td>1.84 ± 0.04</td>
<td>6.40 ± 0.12</td>
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</tbody>
</table>
Curriculum Vitae

Name
Libesha Anparasan

Work Experience

May 2019
Western University: Teaching Assistant - Tropical Marine Environments 3220Z/4257Z

January 2019 - April 2019
Western University: Teaching Assistant - 1002B/1202B Biology

September 2018 - December 2018
Western University: Teaching Assistant - 3338A Biology

January 2018 - July 2018
Western University: Teaching Assistant - 1002B/1202B Biology

January 2018 - April 2018
Western University: Teaching Assistant - 4611B Biology

September 2017 - December 2017
Western University: Teaching Assistant - 2483A Ecology

Publications/Conferences

August 24 - 28, 2019
AOS 2019 Annul Meeting (Poster)

March 22, 2019
Western Graduate Research Forum (Poster)

Awards/Scholarships

June 2019
AOS Student Travel Award
June 2019
AOS Top 20 Student Presentation Finalist

April 2014, 2016, 2017
Dean’s List

September 2013
Western Scholarship of Excellence

**Academic Qualifications**

2017- current
University of Western Ontario (Masters of Biology)

2013 - 2017
University of Western Ontario (Bachelors of Science)

**Volunteer Experience**

November 2018 - May 2019
Science Rendezvous – Programming Committee

February 2019
LAMP Alumni Expo – Biology Graduate Representative

April 2018 - May 2018
Science Rendezvous - Biology Booth Organizer

February 2018
Biology Graduate Research Forum Volunteer