Improving the accuracy of stable isotope analysis in ecological studies of wildlife and helminth parasites

by

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Abstract

Stable isotope analysis (SIA) is increasingly being used to reconstruct wildlife diets and determine trophic structure in ecological communities. These analyses incorporate parameters like diet-tissue discrimination factors (DTDFs) and assumptions about tissue turnover rates that are unknown for many species. Further, DTDFs and tissue turnover rates can be affected by many factors, so it is often unclear whether values derived from different species or under particular conditions (e.g. diet) can be used to generate accurate estimates of wildlife diet and trophic relationships. In my first chapter, we determined the effects of tissue type, diet quality, and stable isotope element on these parameters using controlled-feeding studies with three freshwater snail species. Within tissue type, DTDFs among the three species were similar for certain elements and diets, but also varied in other circumstances. Diet quality had a strong effect on DTDF and even led to some negative DTDFs, which suggested that dietary nutrition sources were being used differently. Hence for my second chapter, we raised snails on diets consisting of different combinations of isotopically distinct ratios of protein:carbohydrate. Using novel equations, we found that the proportion of nutrition sources contributing to synthesized tissue varied by diet quality and snail feeding habits. After better understanding the factors that influence stable isotope ratios, we used this approach in a muskrat-parasite system to determine how diet influenced parasite transmission. We hypothesized that if encounter rates in parasite transmission models were related to diet, then diet estimates for prey and food items involved in parasite life cycles would be positively related to parasite infection patterns. For most parasite species, variation in infection patterns was not well explained by host diet. However, there was an unexpected positive relationship between a plant-encysting trematode and the proportion of snails in the diet, which suggested a new route of transmission. Our results will help other

researchers make more informed decisions about the values they choose to include in SIA models. Further, we demonstrated that by integrating SIA and parasitism, we can determine what food items to sample for SIA models, and fill gaps in our knowledge of parasite life cycles and transmission, which remain poorly understood for most parasite species.

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Thesis format

This thesis is in manuscript format. I wrote Chapters 1, 2 and 3 as individual manuscripts with their own introduction, methods, results, discussions, references, tables and figures. I also wrote an overall introduction and conclusion for my thesis, which summarized background information, conclusions and significance.

Muskrats were provided by fur trappers. I participated in all of the fieldwork related to this research, including sampling prey and plant items for muskrats in three sites in Spring and Fall. I raised and maintained thousands of snails for this research with assistance from undergraduate students working in the laboratory during the summer. I prepared all vegetation and animal muscle samples for stable isotope analysis and performed all other laboratory analyses. I conducted all data analyses and wrote the entire thesis with guidance from my committee.

Thesis Introduction

Stable isotope analysis is becoming a widespread tool in ecology because it estimates the relative contribution of food items in consumers' diet and reveals trophic structure in ecological communities (Peterson and Fry, 1987; Post, 2002; Friesen and Roth, 2016; Hopkins and Kurle, 2016). However, there are parameters in stable isotope models that are unknown for many species and assumptions that are not well tested, which makes the reliability of diet estimates and trophic relationships from stable isotope mixing models varied (Gannes *et al.*, 1997). There were two main goals of my thesis. The first was to perform controlled-feeding studies to understand the factors that influence parameters and assumptions of stable isotope analysis such as diet-tissue discrimination factors (DTDFs), turnover rates, and allocation of nutrition sources to tissue synthesis. The second was to use these parameters and assumptions to perform stable isotope analysis to determine how diet affected parasite transmission in muskrat hosts.

The principle of stable isotope analysis is that the isotopic composition of animal tissue reflects the isotopic composition of the diet for a certain time period. Stable isotope ratios are shown in δ notation in parts per mil (‰): $\delta X = (R_{sample} / R_{standard} - 1)*1000$ where X is the heavier isotope (^{13}C or ^{15}N). R is the ratio of heavier to lighter isotopes ($^{13}C/^{12}C$ or $^{15}N/^{14}N$) in the sample tissue and in the national standard reference material (Vienna Pee Dee Belemnite (VPDB) for carbon and atmospheric N_2 for nitrogen). $\delta^{13}C$ provides information on primary producers because C_3 and C_4 plants have distinctive $\delta^{13}C$ values (Oleary, 1981, 1988) whereas $\delta^{15}N$ estimates the trophic position of consumers because they have approximately a 3‰ enrichment of $\delta^{15}N$ compared to the diet (Peterson and Fry, 1987; Post, 2002). Integrating $\delta^{13}C$ and $\delta^{15}N$ values into Bayesian stable isotope mixing models can reconstruct complex diets (three or more prey/plant items) for wildlife populations over weeks to years depending upon the

species and the tissue being analyzed (Post, 2002; Moore and Semmens, 2008; Parnell *et al.*, 2010). However, small variation in DTDFs could lead to huge differences in diet reconstructions using stable isotope ratios (Ben-David and Schell, 2001; Bond and Diamond, 2011). For example, Bond and Diamond (2011) used DTDFs published for different piscivorous birds to estimate the proportion of krill in a tern's (*Sterna hirundo*) diet and results varied from 10.8% to 90.7%. Consequently, the use of inaccurate parameters in stable isotope mixing models can influence conservation and management decisions (Bond and Diamond, 2011). Although accuracy of DTDFs and tissue turnover times is essential in stable isotope analysis, values for these parameters are unavailable for most species; thus, there have been several calls to increase the number of taxa for which we have this information (Gannes *et al.*, 1997; del Rio *et al.*, 2009).

Diet-tissue discrimination factor (shown as $\Delta^{13}C$ or $\Delta^{15}N$) is a parameter that represents the difference between the isotopic value of the consumer tissue and the diet, and can be influenced by many factors including species (Steinitz *et al.*, 2016), body size (Steinitz *et al.*, 2016), sex (Kurle *et al.*, 2014), tissue type (Tieszen *et al.*, 1983; Arneson and MacAvoy, 2005; Steinitz *et al.*, 2016), digestive physiology (Kurle *et al.*, 2014), and diet quality (Robbins *et al.*, 2005). Turnover rates indicate how quickly the tissue incorporates the isotopic signature of the diet, and are mainly affected by growth and metabolic rates (Tieszen *et al.*, 1983; Hobson and Clark, 1992; MacAvoy *et al.*, 2005; Arneson *et al.*, 2006). These two parameters are best generated from controlled-feeding studies in the laboratory, which can be challenging or impossible to do depending upon the consumer species of interest. Notably, DTDFs and turnover rates are unknown for most invertebrates, which are often primary consumers in food webs and can provide the isotopic baseline for trophic position estimates (Post, 2002).

Another important parameter in stable isotope mixing models is concentration-dependence. This parameter takes into account the concentration of elements in each food item. This parameter is particularly important for omnivores because compared to plants, animal tissues generally have more protein, which is a source of nitrogen. Thus, animal tissues often have smaller C:N ratios than plant tissue (Koch and Phillips, 2002; Phillips and Koch, 2002). Failure to account for the concentration of elements can lead to very different reconstructions of wildlife diet as shown by Phillips and Koch (2002). In models with and without concentration dependence parameters, the estimated proportion of salmon in brown bear's diet varied from 59% to 26%, respectively. Thus, concentration dependence should be estimated for each food item, but additional factors such as digestion and assimilation rates are also important. Dietary macromolecules (i.e., carbohydrate, protein and lipid) may be routed differently in consumer tissues, thus causing variation in assimilation rates. It is hypothesized that dietary carbohydrates are mainly oxidized to produce energy or converted to glycogen to store energy for later use (Hobson and Stirling, 1997). On the other hand, dietary protein may be mainly used in tissue replacement and synthesis (Tieszen and Fagre, 1993; MacAvoy *et al.*, 2005). Thus, these two dietary sources may be incorporated into tissues at different rates.

Incorporation rates are important to understand because they can help explain variation in DTDFs. Animal tissues generally have higher stable isotope ratios than their diet (positive DTDF values) because lighter isotopes move more rapidly through biochemical pathways and thus molecules containing lighter isotopes are more easily used for energy (Peterson and Fry, 1987). However, negative DTDFs have been occasionally observed, which is thought to occur when dietary nutrition sources with distinct isotopic values are used differently in the tissue (MacAvoy *et al.*, 2005). Quantifying the contribution of different dietary nutrition sources to animal tissues

can test this assumption. If the contribution of dietary macromolecules to tissue synthesis differs among tissue types and organisms, this would also explain why DTDFs vary by tissue and species. Very few studies have quantified incorporation rates despite their influence on concentration dependence and DTDFs.

Another basic assumption in stable isotope analysis is that all potential food items are sampled, which is hard to meet especially for wildlife with complex diets. One approach to confirm what food items should be sampled is to identify parasites within the consumer and use knowledge of their life cycles to infer what food item(s) was consumed. Trophically-transmitted parasites serve as indicators of diet because the host becomes infected when consuming infected plant or animal tissue. Many vertebrates become infected with adult trematodes after eating larval trematodes that were encysted on the surface of plants or within the tissues of intermediate animal hosts such as snails, fish, frogs, or arthropods (Johnson and McKenzie, 2009). However, all food items may not be identified using parasites because knowledge of parasite life cycles and transmission is often incomplete (Bolek *et al.*, 2016).

Diet reconstruction using stable isotope ratios could fill gaps in our knowledge about parasite life cycles by indicating potential hosts in the life cycle. Often life cycles are determined by a few studies conducted in a limited context (Bolek *et al.*, 2016). For example, life cycles may only be established for part of the parasite's range, and not all potential hosts in that area, much less across that parasite's entire range, may be investigated for parasite infection. Relying on field data alone can also be misleading as laboratory investigations of life cycles have revealed that when ecological barriers are removed, additional hosts are involved (Detwiler and Janovy, 2008). Diet reconstruction could suggest what hosts should be targeted for parasite investigation and could reveal if life cycles vary seasonally or according to geography. In addition, the

proportion of food items in a diet may improve parasite transmission models by aiding in quantifying the "encounter" parameter (i.e. β in Anderson and May, 1979). In transmission models, encounter could take into account the proportion of particular food items from stable isotope analysis. By integrating stable isotope analysis and knowledge of parasite life cycles, we can address pitfalls associated with estimating wildlife diet, parasite life cycles and transmission.

My thesis consists of three chapters. Chapter 1 examined carbon and nitrogen DTDFs and turnover rates in three freshwater snail species and tested how tissue type, diet quality, and stable isotope element influenced these parameters. In Chapter 2, we quantified the contribution of dietary nutrition sources to tissue synthesis to better explain the variation in DTDFs. Chapter 3 integrated diet estimates from stable isotope analysis and parasite infection patterns to improve our understanding of wildlife diet, parasite life cycles and parasite transmission.

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Chapter 1: Carbon and nitrogen isotopic turnover rates and diet-tissue discrimination vary depending upon diet in freshwater snail species

Introduction

The use of stable isotope ratios to reconstruct consumers' diets and determine trophic structure in ecological communities is becoming widespread (Peterson and Fry, 1987; Post, 2002). The rationale behind stable isotope analysis is that the isotopic composition of animal tissue reflects their diet's isotopic composition for a certain time period. Carbon stable isotope ratios ($^{13}C/^{12}C$ or $\delta^{13}C$) provide information on the primary producer in the food web of the animal because C_3 and C_4 plants have distinctive $\delta^{13}C$ values (Oleary, 1981, 1988); nitrogen stable isotopic values ($^{15}N/^{14}N$ or $\delta^{15}N$) can be used to estimate the trophic position because consumers have approximately a 3‰ enrichment of $\delta^{15}N$ compared to the diet (Peterson and Fry, 1987; Post, 2002). Ratios from these two elements are integrated into Bayesian stable isotope mixing models to reconstruct complex diets (three or more prey/plant items) for wildlife populations over weeks to years depending upon the species and the tissue being analyzed (Post, 2002; Moore and Semmens, 2008; Parnell *et al.*, 2010). The accuracy of diet estimates from these models is influenced by parameters including diet-tissue discrimination factors (DTDFs, also called "trophic discrimination factors", "trophic enrichment factors" or "fractionation factors") and tissue turnover rates (Gannes *et al.*, 1997; Phillips and Gregg, 2001). However, values for these parameters are unavailable for most species, prompting several calls to increase the number of taxa for which we have this information (Gannes *et al.*, 1997; del Rio *et al.*, 2009).

The use of stable isotopes to study the diet is based on the principle that animal tissues generally have a fixed isotopic enrichment or depletion compared to their diet. DTDF (shown as $\Delta^{13}C$ or $\Delta^{15}N$) is the parameter that describes this isotopic ratio difference ($\Delta^{13}C = \delta^{13}C_{tissue}$ -

$\delta^{13}C_{diet}$). Studies often assume 0.5‰ to 1‰ for $\Delta^{13}C$ and 3.4‰ for $\Delta^{15}N$ (Minagawa and Wada, 1984; Post, 2002). However, because DTDFs are related to the biochemical reaction of an organism's tissue in a certain circumstance, many factors can affect DTDFs including species (Steinitz *et al.*, 2016), body size (Steinitz *et al.*, 2016), sex (Kurle *et al.*, 2014), tissue type (Tieszen *et al.*, 1983; Arneson and MacAvoy, 2005; Steinitz *et al.*, 2016), digestive physiology (Kurle *et al.*, 2014), and diet quality (Robbins *et al.*, 2005). For example, diet quality influences DTDFs because of differences in the type and amount of amino acids in animal and plant tissues (Robbins, 1993; Roth and Hobson, 2000; Hoffman and Falvo, 2004). Animal tissues are higher in diet quality because their proteins can be broken down into more amino acids than plant-based proteins (Hoffman and Falvo, 2004). For instance, animal tissue provides all essential amino acids that humans cannot derive on their own, whereas plant sources always lack one or more essential amino acids. Moreover, animal tissues are generally higher in protein content than plant tissues because animal cell walls are mainly protein, whereas plant cell walls consist mainly of carbohydrate (Robbins, 1993). These characteristics suggest that the higher the proportion of animal protein in the diet, the higher the diet quality (Robbins *et al.*, 2005). Herbivores have a plant diet that is lower quality because they need to derive more amino acids from other dietary sources (i.e., carbohydrates) compared to consumers that eat animal-derived protein. Because protein is generally enriched in ^{13}C up to 3‰ relative to carbohydrates, herbivores will have a higher DTDF than carnivores (DeNiro and Epstein, 1978).

Another main parameter, turnover rate, indicates how quickly the tissue shows the isotopic signature of the diet, and is mainly affected by metabolic rates (Tieszen *et al.*, 1983; Hobson and Clark, 1992; MacAvoy *et al.*, 2005; Arneson *et al.*, 2006). Metabolism among tissues can be different because of the rate at which old tissues are replaced, especially for

protein metabolism. For example, more metabolically demanding tissues like liver reflect the diet within a few weeks, but tissues with lower metabolic rates like muscle reflect the diet from the previous few months (Tieszen *et al.*, 1983; Arneson *et al.*, 2006). Many animals are still growing when they are incorporating their diet into their tissues, so measurements of turnover rates may also be affected by growth, which involves new tissue synthesis (e.g. somatic growth).

Given the number of factors that can affect DTDFs and turnover rates, these two parameters are best estimated from controlled-feeding studies that use known diets in a consistent environment for a sufficient time (e.g. Tieszen *et al.*, 1983; Arneson and MacAvoy, 2005; Barquete *et al.*, 2013; Steinitz *et al.*, 2016). However, these studies can be challenging or impossible to do depending upon the consumer species of interest, and thus, DTDFs and turnover rates for most species are unknown. As a result, many stable isotope studies make assumptions about turnover rates and discrimination factors from other species that are phylogenetically or trophically similar (e.g. Hersey *et al.*, 2013; Friesen and Roth, 2016; Hopkins and Kurle, 2016). However, small differences in DTDFs could lead to large differences in diet reconstruction from stable isotope ratios (Ben-David and Schell, 2001; Bond and Diamond, 2011). For example, Bond and Diamond (2011) used DTDFs published for different piscivorous birds to estimate the proportion of krill in a tern's (*Sterna hirundo*) diet and depending upon the DTDF the results varied from 10.8% to 90.7%. Consequently, the use of inaccurate parameters in stable isotope mixing models could influence conservation and management decisions (Bond and Diamond, 2011).

As a result of these issues, several reviews have called attention to the lack of laboratory-based feeding experiments that are essential for accurate estimates of DTDF and turnover rates (Gannes *et al.*, 1997; del Rio *et al.*, 2009). Although the number of such studies has increased

since the 1990s, del Rio *et al.* (2009) concluded that more studies were needed from consumers in more ecosystems, across food webs, for specific taxa such as invertebrates, and even for specific tissues. Invertebrates are often primary consumers in food webs thus knowing DTDFs and turnover rates of invertebrates can provide the isotopic baseline for trophic position estimates (Post, 2002). However, there are fewer than 93 published pairs (i.e. for C and N) of DTDFs for invertebrates from 44 species (Caut *et al.*, 2009), and fewer than 60 published pairs of half-life for invertebrates (Vander Zanden *et al.*, 2015). Half-life is often estimated as a representation of turnover rate in stable isotope studies, and is defined as the time it takes for 50% of the stable isotopes in the tissue to be replaced by the stable isotopes in the diet. In most cases, the whole organism was used to generate DTDFs and half-life for invertebrates (84/93 cases for DTDFs and 44/60 cases for turnover rate estimates); thus, tissue specific estimates are scarce. Of these invertebrate studies, only a third were conducted on aquatic species and five of those were freshwater species.

We focused on freshwater snails because they are ubiquitous in freshwater ecosystems and are primary consumers that alter the amount of detritus, periphyton (a complex mixture of algae, cyanobacteria, heterotrophic microbes, and detritus) and algae in wetlands, streams, and lakes (Brown, 2001). In addition, snails play a key role in disease transmission in freshwater ecosystems because they are hosts for many parasites including trematodes. Differences in diet among snail species could reflect microhabitat use, which could then be used to predict where hot spots of transmission to wildlife could occur. Moreover, knowing the DTDF and turnover rates for snails provides an opportunity to test how parasitism influences snail host diet.

The objective of this study was to determine DTDFs and turnover rates of carbon and nitrogen stable isotope ratios in muscle and gonad of three freshwater snail species (*Helisoma*

trivolvis, *Lymnaea elodes*, and *Lymnaea stagnalis*) raised in the laboratory. We manipulated diet by switching from a low-protein to a high-protein diet because they differed in their stable isotope ratios. The high-protein diet had a higher isotopic value for both carbon and nitrogen compared to the low-protein diet. Differences in DTDFs and turnover rates among species were predicted because of trophic differences (*H. trivolvis* = generalist feeder; *L. elodes* = algae and carrion specialist; *L. stagnalis* = herbivore feeding primarily on macrophytes and algae) (Brown, 1982; Rybak, 2016). We predicted that the herbivore (*L. stagnalis*) would have higher DTDFs compared to the omnivores (*H. trivolvis* and *L. elodes*). Due to the trophic differences among the species, we also predicted that diet quality would affect snail size. The generalist feeder *H. trivolvis* would grow larger on high-protein diet compared to low-protein diet, whereas the primarily plant-feeding *Lymnaea* spp. would grow larger on low-protein diet compared to high-protein diet. We predicted that snail gonad tissue would have lower DTDFs and faster turnover rates compared to muscle tissue. Gonads are considered more metabolically active tissues since egg-laying activity starts around two months of age for all three species, and gonadal maturation and gonaduct functions are required for egg production (Janse *et al.*, 1989; Norton and Bronson, 2006; Morishita *et al.*, 2010). By determining these parameters, we can more accurately estimate trophic positions in food webs involving aquatic snails and test how diet preference affect DTDF and turnover rates in wildlife diet reconstruction.

Methods

Laboratory diets

The high-protein diet was based on a recipe by Sandland and Minchella (2003). We added ^{13}C-enriched item (corn powder) to ensure that the turnover of carbon isotopic value was detectable. The diet consisted of 1 g BactoAgar (powder derived from algae), 1 g corn powder

and 10 g Tetramin RichMix (40% protein, 5% fat, 2% fiber, 6% moisture, 1.3% phosphorous) mixed with 83 mL of boiled water. The mixture was poured into a tray (0.5 cm depth) and placed in a drying oven at 60 ℃ for 12 hours to prevent the growth of bacteria. The low-protein diet consisted of well-rinsed green leaf lettuce purchased from a local grocery store.

Snail sampling

For each species of snail, 450 hatchlings from laboratory breeding colonies were raised individually in separate 100 mL glass jars with 80 mL of well water. All snails were maintained in a room with a 12h:12h light/dark scheme. Each individual was fed green leaf lettuce *ad libitum* for ~10 weeks until shell length was approximately 10 mm (from apex to aperture, Burch, 1982). Then the snails were randomly assigned into a control or experimental group (n = 225 for each). Control snails continued their lettuce (low-protein) diet, while the experimental snails were switched to the high-protein diet. Snails from both groups were subsampled at 1, 2, 4, 8, 16, 32 and 48 days post-diet change for *H. trivolvis* and *L. elodes*. Additional subsampling for *L. stagnalis* included 64 and 80days post diet-change because 48 days was not sufficient for the related species *L. elodes* to achieve turnover (see Results). For each subsampling, 30 snails from each group were randomly chosen. Snail length for each individual was measured to the nearest 0.01 mm to assess snail size. Muscle (foot) and gonads from ten snails were each pooled into one sample to ensure that there was enough tissue for stable isotope analysis (three replicates per subsample). Tissue samples were frozen at -20 ℃.

Sample processing for stable isotope analysis

Snail tissue samples and high-protein diet samples were processed by freeze drying for 48 hours, and then ground to a fine powder with a mortal and pestle. We extracted lipids from

the snail and diet samples because lipids have different carbon isotopic composition than other components, which could lead to high variation in δ^{13}C measurement for samples with different amount of lipids (Rau *et al.*, 1992). Lipids were removed from samples with petroleum ether for 16 hours using a Soxhlet apparatus, and then oven dried at 60 ºC for 24 hours (Elliott *et al.*, 2017). Low-protein (lettuce) samples were placed in a drying oven at 60 ºC for 48 hours and then ball-milled to a fine powder. The powdered samples were weighed (0.4–0.6 mg for snail tissue and high-protein diet; 2.5–3 mg for low-protein diet) and sent to the Chemical Tracers lab at the Great Lakes Institute of Environmental Research (GLIER), University of Windsor, Stratford, Ontario and processed with an Isotope Ratio Mass Spectrometer (Thermo Delta V) to determine carbon and nitrogen stable isotope ratios.

Data analysis

To show the turnover pattern of carbon and nitrogen isotopic values, exponential equations were used (Tieszen *et al.*, 1983; Hobson and Clark, 1992). δ^{13}C and δ^{15}N values of tissue were fitted to equation:

$$\delta^{13}\text{C or } \delta^{15}\text{N} = \delta_n + (\delta_0 - \delta_n)\text{e}^{-\lambda t} \qquad (1)$$

where δ_n represents the isotopic value at equilibrium, δ_0 represents the δ value prior to the diet switch, λ is the turnover rate (%/day) derived from the model, and t is the time of feeding with the new diet (days). Half-life was calculated as ln(2)/λ, which is the time (days) it takes for 50% of the tissue to be replaced. We also calculated the time it takes for 99.99% of tissue to be replaced as ln(10000)/λ as recommended by Tieszen *et al.* (1983).

Δ^{13}C and Δ^{15}N were calculated for each snail species (*H. trivolvis*, *L. elodes*, *L. stagnalis*) and tissue type (muscle and gonad) by comparing the mean diet isotopic value and δ_n in Equation 1 for snails fed on high-protein diet. For the low-protein diet, DTDFs were calculated by

subtracting the mean isotopic value of the snail tissue in all sample days and the mean isotopic value of the diet. The SD of DTDFs were calculated with the following equation:

$$SD_{DTDF} = \sqrt{SD_{tissue}^2 + SD_{diet}^2}$$

Two-sample t-tests determined whether $\Delta^{13}C$ and $\Delta^{15}N$ values were different between the diet and tissue types.

Two general linear models were used to determine the influence of species, diet, time and tissue on $\delta^{13}C$ and $\delta^{15}N$ values, respectively. An additional general linear model was used to determine the effect of snail species, diet and time on size (shell length). Model selection began with consideration of a full model including all main effects, covariates, and interactions terms. Components were dropped if they were not significant ($P > 0.05$) and the Akaike information criterion (AIC) value was lower without the component in the model (Crawley, 2012). All statistics were performed in R version 3.2.5 (R Core Team, 2016) using packages MASS (Venables and Ripley, 2002).

Results

Snails fed with high-protein diet had lower $\Delta^{13}C$ ($t = 3.20$, $df = 10$, $P = 0.009$) and $\Delta^{15}N$ values ($t = 3.31$, $df = 10$, $P = 0.008$) than those fed on low-protein diet regardless of tissue types (Tables 1.1 and 1.2). When fed on low-protein diet, $\Delta^{13}C$ and $\Delta^{15}N$ values were positive for both muscle and gonad tissue in all three snail species. On the high-protein diet, $\Delta^{13}C$ values were positive except for negative $\Delta^{13}C$ values in both tissues for *L. elodes*. Negative $\Delta^{15}N$ values were observed in the gonad tissues for all three species, and the muscle tissue of *H. trivolvis*.

Muscle tissue had higher DTDFs than gonad tissues (Table 1.1 and 1.2). Two-sample t-tests showed no difference in DTDFs between tissue types ($\Delta^{13}C$: $t = 1.64$, $df = 10$, $P = 0.133$;

$\Delta^{15}N$: t = 1.58, df = 10, $P = 0.146$). The lack of significance may be due to low statistical power and because average differences between tissues is confounded by diet. The differences in the average values suggest that there are some differences between the tissues with biological relevance. For example, on the low-protein diet, the average $\Delta^{13}C$ for muscle was 2.48‰, but the values for gonad on the low-protein diet, and gonad and muscle on the high-protein diet were more similar (0.91‰, 0.38‰ and 0.03‰, respectively; Table 1.1). For $\Delta^{15}N$, the average values for muscle and gonad tissue on the low-protein diet were 3.39‰ and 1.60‰, respectively; on the high-protein diet, the average $\Delta^{13}C$ of muscle and gonad tissue were 0.55‰ and -1.05‰, respectively (Table 1.2).

Species had similar $\Delta^{13}C$ on the low-protein diet in both tissue types (maximum difference < 0.2‰). In contrast, $\Delta^{13}C$ among species within tissue types varied more widely on the high-protein diet (maximum difference > 1.1‰). For $\Delta^{15}N$, species had similar values on high-protein diet in both tissue types (maximum difference < 1.2‰) and different values on the low-protein diet in both tissue types (maximum difference > 3.0‰).

Diet ($F_{1,245} = 2475.91$, $P < 0.001$), time ($F_{1,245} = 252.22$, $P < 0.001$) and tissue type ($F_{1,245} = 125.92$, $P < 0.001$) explained most of the variation in $\delta^{13}C$ values ($r^2 = 0.93$), but there was no effect of snail species ($F_{2,245} = 0.21$, $P = 0.81$; Table 1.3). For the model of $\delta^{15}N$, all factors were significant ($P < 0.001$; $r^2 = 0.86$; Table 1.3).

Snail stable isotope ratios began changing within days after switching the diet (Figures 1.1 and 1.2). Snail gonad tissue had faster turnover rates than muscle tissue for both carbon (half-life: 5–5.5 days vs. 6.1–8 days) and nitrogen (half-life: 2.4–5.1 days vs 2.2–13.3 days) isotope ratios. The half-life of $\delta^{13}C$ for each tissue type was similar among species (max difference of 1.9 days for muscle, 1.5 days for gonad). Variation in half-life within tissue among species was

greater for $\delta^{15}N$ (max difference of 11.1 days for muscle, 2.7 days for gonad) compared to $\delta^{13}C$. The differences among species and tissue were more apparent with the 99.99% turnover time. In some cases, snail tissue achieved nearly complete turnover in approximately one month; in other cases, a much longer time span exceeding 100 days was required, which in the laboratory and nature could be greater than the lifetime of a snail. Carbon and nitrogen reflected different time periods of diet in snail species. The half-life of $\delta^{13}C$ (up to 6.4 days) was shorter than those of $\delta^{15}N$ (up to 13.3 days) for *H. trivolvis* and *L. elodes*. In contrast, *L. stagnalis* $\delta^{15}N$ (mean = 2.3 days) had a shorter half-life compared to $\delta^{13}C$ (mean = 6.6 days).

Species differed in size ($F_{2,245} = 721.96$, $P < 0.001$) which could have occurred because diets were not switched for all species at exactly the same length. *Helisoma trivolvis* and *L. elodes* were on average ~10 mm, while *L. stagnalis* was ~14 mm when the diet was switched. However, the best model also included a significant species:day interaction ($F_{2,245} = 319.36$, $P < 0.001$) suggesting that differences in growth rate among the species varied over time (Figure 1.3). During the course of 48 days, *H. trivolvis* increased in growth (mean from two diets ± SE) by 37% (10.02 ± 0.13 to 13.72 ± 0.29 mm), while *L. elodes* and *L. stagnalis* increased by 27% (10.13 ± 0.16 to 12.88 ± 0.32 mm), and 26% (13.44 ± 0.20 mm to 17.00 ± 1.08 mm), respectively.

Diet affected size as a fixed factor ($F_{1,245} = 6.14$, $P = 0.014$) and as part of an interaction with species:diet:day ($F_{3,245} = 149.55$, $P < 0.001$). This interaction indicates that after accounting for the fact that all snails would grow larger during the experiment, snail species were different sizes depending upon the diet. Most importantly, diet affected growth within a species; *Lymnaea* spp. grew larger on the low-protein diet compared to the high-protein diet while *H. trivolvis* grew larger on the high-protein diet compared to the low-protein diet (Figure 1.3).

Discussion

Estimates of diet-tissue discrimination factors and isotopic turnover rates from controlled-feeding studies are essential for ensuring that stable isotope analysis accurately reflects wildlife diet and trophic relationships. Using freshwater snails, we found that diet affected DTDFs. For some diets and elements, all species had similar DTDFs, but for other diets and elements, DTDFs could be different among species. Turnover rates were similar among species within tissue type for carbon, but more variation among species was observed for nitrogen. In addition, growth among species varied depending on diet; some species grew larger on high-protein (i.e. *H. trivolvis*) while others grew larger on low-protein diet (i.e. *Lymnaea* spp.). In turn, differences among species in growth influenced turnover rates (i.e. turnover was faster in the species with the highest growth rate when the diet was switched from low to high-protein. This result supports the hypothesis that growth is one of the main processes that affects tissue turnover (Fry and Arnold, 1982). Interestingly, this relationship was not true for DTDFs, where the pattern was the same for all species regardless of growth performance (low-protein > high-protein). This finding suggests that the same factor (i.e. diet) might influence two key parameters of stable isotope analysis differently.

Our study found a similar range and mean of $\Delta^{13}C$ values compared to other studies of aquatic organisms. We observed a slightly smaller range of $\Delta^{13}C$ values (-1.16–+2.49‰) relative to the range (-2.1–+2.8‰) reported for 14 aquatic invertebrates species that were sampled from freshwater and marine environments (Vander Zanden and Rasmussen, 2001). The mean $\Delta^{13}C$ values from snails fed the low-protein diet (1.52‰) were similar to the mean of 1.33‰ for freshwater aquatic organisms (n = 42 studies of birds, fish and invertebrates) reported by Caut *et*

al. (2009). This similarity in $\Delta^{13}C$ suggests that if these values are unknown, the mean value could be used to predict the diet of freshwater aquatic organisms from stable isotope analysis.

It remains unclear if the same conclusions can be made about $\Delta^{15}N$. Post (2002) recommended that animal studies should use 3.4‰, which was an average value derived from 56 studies that included a range of animals from copepods to polar bears that were sampled from different environments (i.e. freshwater, marine and terrestrial). In our study, most of the $\Delta^{15}N$ values were relatively smaller with a total average of 1.28‰, which shows that using Post's value could lead to inaccurate diet estimates depending upon the taxon. Previous studies with three gastropod species (two marine and one terrestrial) reported a mean of -0.06‰ whereas snail species from our study had a combined mean value of 0.04‰ when fed a diet with similar $\delta^{15}N$ values to the previous studies (high-protein diet) (DeNiro and Epstein, 1981; Kurata *et al.*, 2001). This result indicates that mean $\Delta^{15}N$ values from phylogenetically related species with similar diets can be useful for diet reconstruction using stable isotope analysis.

Snails fed diets with higher $\delta^{13}C$ and $\delta^{15}N$ values (i.e., high-protein diet) had lower DTDFs than those fed on diets with lower isotopic ratios (i.e., low-protein diet) (Table 1.1). Our results are consistent with other studies that included a range of taxa including mammals, birds, fishes and invertebrates (see reviews by Caut *et al.*, 2009; Caut *et al.*, 2010). Robbins *et al.* (2005) predicted that $\Delta^{15}N$ values should decrease as diet quality increases with trophic level. This relationship has been observed in some types of consumers. For example, carnivores that ingested higher quality protein had lower $\Delta^{15}N$ values than herbivores (Robbins *et al.*, 2005). In our study, we confirmed that for the same consumer species, diets with higher quality had lower $\Delta^{15}N$ values. Variation in DTDF by diet suggests that it is important to choose DTDFs generated by controlled-feeding studies that mimic diets in the wild. Otherwise, the diet estimates from

stable isotope mixing models may not be accurate. These results suggest that different DTDFs should be applied to diet items that differ in protein content such as between animal prey and plants. This consideration could be important for omnivorous consumers that eat a mixture of animal and plant tissues that vary in protein content, but even for consumers whose diet may be subject to changes in protein quality over space and time.

We also observed negative $\Delta^{13}C$ values for snails fed on the high-protein diet, although animal tissue is generally enriched in ^{13}C compared to the diet (Peterson and Fry, 1987). Negative values could occur if carbohydrates with higher isotopic values than other dietary nutrition sources (e.g., protein) were mainly used as energy, instead of being incorporated into the tissue. In some controlled-feeding studies, mice were fed a combination of carbohydrate (cane sucrose) and protein (casein) that had distinctly different $\delta^{13}C$ values (Arneson and MacAvoy, 2005; MacAvoy *et al.*, 2005; Kurle *et al.*, 2014). Mice preferentially used protein to make new tissue components rather than carbohydrates, with roughly 75% of the carbon in the tissue being derived from protein (MacAvoy *et al.*, 2005). Our high-protein diet had soybean and corn, which are both C_4 plants with high $\delta^{13}C$ values, potentially giving our high-protein diet a higher $\delta^{13}C$ value. Negative $\Delta^{13}C$ for snails on high-protein diet could also have occurred if most carbohydrate was lost as CO_2 after being used as energy as hypothesized by Hobson and Stirling (1997).

Our study also suggests that tissue type can influence DTDFs and turnover rates. As predicted, we found that more metabolically active tissue (i.e. gonad) tended to more closely reflect the isotopic value of the diet (mean $\Delta^{13}C = 0.29‰$ and $\Delta^{15}N = 0.43‰$) compared to muscle tissue (mean $\Delta^{13}C = 1.35‰$ and $\Delta^{15}N = 2.13‰$) and had shorter half-life (mean = 4.4 days) compared to muscle tissue (mean = 7.5 days). Differences among tissues types in DTDF

and turnover rates are well-tested in vertebrates (Tieszen *et al.*, 1983; Boecklen *et al.*, 2011), but most stable isotope studies of invertebrates used the whole organism (DeNiro and Epstein, 1978; DeNiro and Epstein, 1981; Kurata *et al.*, 2001). Our study suggested that metabolic activity of tissue types could influence DTDFs and turnover rates in invertebrates, though no statistically significant differences among tissues were found likely due to a small sample size (n = 12, 2 diets × 2 tissue types × 3 replicates). However, gonad tissue reflected the diet in about half the time as muscle tissue. Thus, future studies should consider using a certain tissue and tissue specific values for invertebrates in stable isotope analysis just as studies of vertebrates do.

In general, freshwater snails had fast turnover rates, with half-life < 7 days for $\delta^{13}C$ and < 14 days for $\delta^{15}N$. If diet reflects environmental change, then diet studies of snails could indicate environmental changes over short time scales in wetlands. We also found that $\delta^{13}C$ and $\delta^{15}N$ had distinctive turnover rates as carbon had a quicker turnover rate than nitrogen in the foot tissue of *L. elodes* and *H. trivolvis*. Differences between the elements could occur because carbon is derived from both carbohydrates and proteins whereas nitrogen only comes from protein in the diet (Kurle, 2009). The difference in turnover rates between C and N suggests that future studies should focus on a single tissue but use different elements to better understand the temporal variation in ecosystems by estimating diet at different time periods. For example, $\delta^{13}C$ values can be used to determine if recent diet differs, whereas $\delta^{15}N$ values can be used to determine if diet differs over longer time periods. This approach could also be used to understand how diet changes during migration and differs among seasons.

In addition to affecting DTDFs and turnover, diet also affected growth. On the low-protein diet, all snail species had similar growth rates. However, on the high-protein diet, some species grew larger (i.e. *H. trivolvis*) while others grew less (i.e. *L. elodes* and *L. stagnalis*). In

addition to increased growth, turnover rates for $\delta^{13}C$ were faster on the diet for the species that achieved the greatest growth. For example, *H. trivolvis* had quicker turnover rates than *Lymnaea* spp. when snails were fed the high-protein diet.

Despite repeated calls for more studies, controlled-feeding studies remain a minor but essential component in stable isotope analysis. Expanding the number of taxa will make estimates of DTDFs and turnover rates more accurate, which will in turn allow investigators to inform models that estimate wildlife diet and trophic relationships. This study is the first to examine these two parameters with both carbon and nitrogen in freshwater gastropods. Our study suggests that it is best to estimate DTDF and turnover for species especially if the diet may vary in its quality over space or time. If it is unrealistic to perform controlled-feeding studies using a diet that approximates the diet in the wild, $\Delta^{13}C$ for freshwater animals is best predicted by using an average value from species living in a freshwater environment. In contrast, the larger variation in $\Delta^{15}N$ suggests that it is best predicted from averaging values from several closely related species with similar diets. Moreover, our results suggest invertebrates are similar to vertebrates in that tissue-specific values should be considered when estimating DTDF and turnover rates. The most significant finding from our study is that diet affected growth performance, which in turn affected DTDFs and turnover rates. This result has significant implications for field studies of wildlife diet. In nature, the preferred diet may not always be available, suggesting that DTDFs and turnover rates may change depending on the season and food availability.

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Table 1.1. Mean carbon isotopic signatures (± SD) and diet tissue discrimination factors (DTDFs) according to species, tissue type and diet (‰) from all sample days for low-protein diet, and 48 or 80 days after diet was switched for high-protein diet.

		Low-protein diet			High-protein diet		
Species[a]	**Tissue**	**N**	**$\delta^{13}C$**[b]	**$\Delta^{13}C$**[c]	**N**	**$\delta^{13}C$**	**$\Delta^{13}C$**
Diet for *H. trivolvis*		4	-28.43 ± 0.71	-	2	-19.32 ± 0.30	-
H. trivolvis	**Muscle**	21	-25.96 ± 0.32	2.47 ± 0.78	3	-18.79 ± 0.11	0.53 ± 0.11
H. trivolvis	**Gonad**	21	-27.46 ± 0.32	0.97 ± 0.78	3	-19.1 ± 0.10	0.22 ± 0.32
Diet for *L. elodes*		5	-27.84 ± 1.31	-	2	-19.44 ± 0.23	-
L. elodes	**Muscle**	20	-25.32 ± 0.53	2.52 ± 1.41	3	-20.1 ± 0.63	-0.66 ± 0.67
L. elodes	**Gonad**	20	-26.90 ± 0.56	0.94 ± 1.42	3	-20.06 ± 0.43	-0.62 ± 0.49
Diet for *L. stagnalis*		3	-27.92 ± 1.36	-	2	-19.6 ± 0.10	-
L. stagnalis	**Muscle**	24	-25.48 ± 0.30	2.44 ± 1.39	2	-18.32 ± 0.69	1.28 ± 0.70
L. stagnalis	**Gonad**	24	-27.10 ± 0.25	0.82 ± 1.38	2	-19.11 ± 0.25	0.49 ± 0.27

Mean carbon isotope signatures (± SD) were also obtained for each diet for each species.

[a]*Helisoma trivolvis* (48 days), *Lymnaea elodes* (48 days), *Lymnaea stagnalis* (80 days)

[b]Stable isotope signature

[c]Diet-tissue discrimination factor

Table 1.2. Mean nitrogen stable isotopic signatures (± SD) and diet-tissue discrimination factors (DTDFs) according to species, tissue type and diet (‰) from all sample days for low-protein diet, and 48 or 80 days after diet was switched for high-protein diet

		Low-protein diet			High-protein diet		
Species[a]	**Tissue**	**N**	**$\delta^{15}N$[b]**	**$\Delta^{15}N$[c]**	**N**	**$\delta^{15}N$**	**$\Delta^{15}N$**
Diet for *H. trivolvis*		4	3.45 ± 1.76	-	2	8.86 ± 0.92	-
H. trivolvis	**Muscle**	21	5.30 ± 0.42	1.85 ±1.81	3	8.81 ± 0.06	-0.05 ± 0.92
H. trivolvis	**Gonad**	21	3.66 ± 0.49	0.21 ± 1.83	3	7.64 ± 0.05	-1.22 ± 0.92
Diet for *L. elodes*		5	2.57 ± 2.10	-	2	8.70 ± 0.37	-
L. elodes	**Muscle**	20	5.62 ± 0.67	3.05 ± 2.20	3	9.24 ± 0.29	0.54 ± 0.47
L. elodes	**Gonad**	20	3.91 ± 0.85	1.34 ± 2.27	3	7.25 ± 0.55	-1.45 ± 0.66
Diet for *L. stagnalis*		3	-1.88 ± 2.88	-	2	8.43 ± 0.14	-
L. stagnalis	**Muscle**	24	3.40 ± 1.99	5.28 ± 3.50	2	9.58 ± 0.10	1.15 ± 0.17
L. stagnalis	**Gonad**	24	1.37 ± 1.96	3.25 ± 3.48	2	7.95 ± 0.12	-0.48 ± 0.18

Mean nitrogen isotope signatures (± SD) were also obtained for each diet for each species.

[a]*Helisoma trivolvis* (48 days), *Lymnaea elodes* (48 days), *Lymnaea stagnalis* (80 days)

[b]Stable isotope signature

[c]Diet-tissue discrimination factor

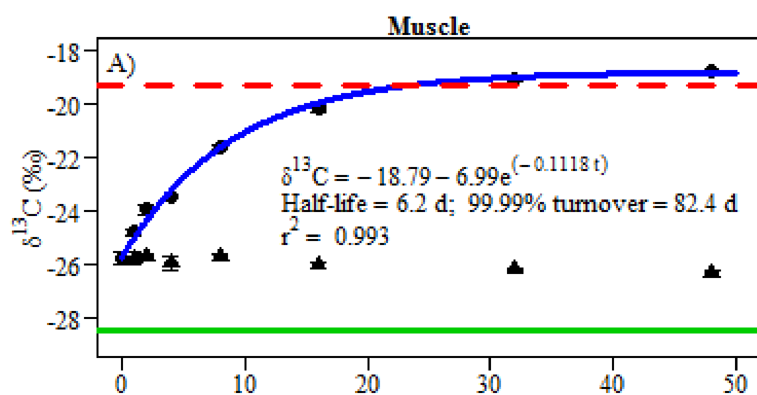
Table 1.3. General linear model results for the effect of species, diet, time, tissue, and significant interactions on $\delta^{13}C$ and $\delta^{15}N$ values.

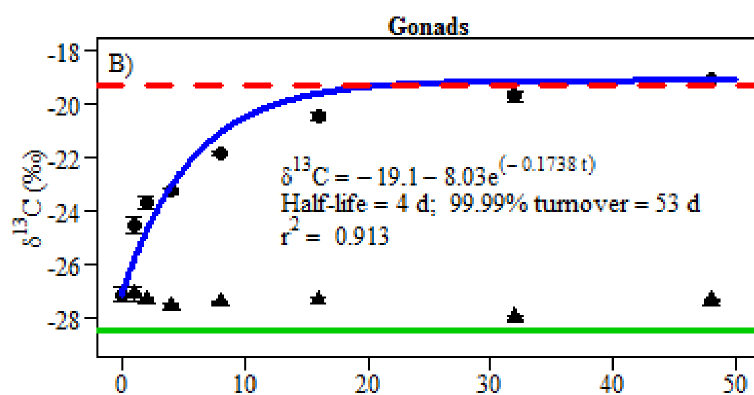
	$\delta^{13}C$			$\delta^{15}N$		
Factors	df	*F*	*P*	df	*F*	*P*
Species[a]	2, 243	0.21	0.806	2, 243	18.02	< 0.001
Diet[b]	1, 242	2475.91	< 0.001	1, 242	787.09	< 0.001
Time	1, 241	252.22	< 0.001	1, 241	20.27	< 0.001
Tissue[c]	1, 240	125.92	< 0.001	1, 240	180.71	< 0.001
Species × Diet	2, 238	16.00	< 0.001	2, 238	64.31	< 0.001
Species × Time	-	-	-	2, 236	27.22	< 0.001
Diet × Time	1, 237	435.09	< 0.001	1, 235	197.00	< 0.001
Diet × Tissue	1, 236	43.91	< 0.001	1, 234	16.05	< 0.001
Species × Diet × Time	-	-	-	2, 232	16.69	< 0.001

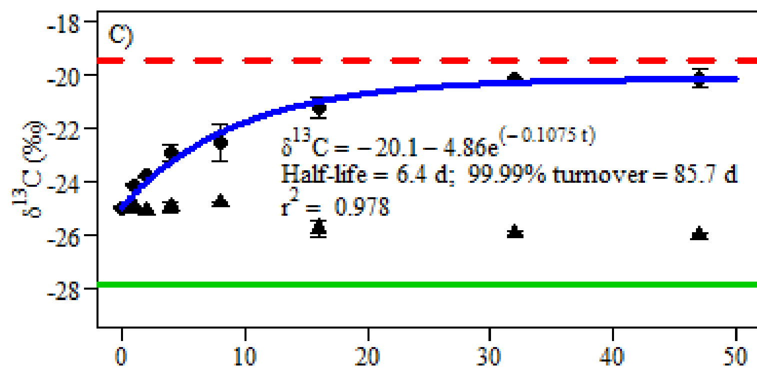
[a]*Helisoma trivolvis*, *Lymnaea elodes*, *Lymnaea stagnalis*

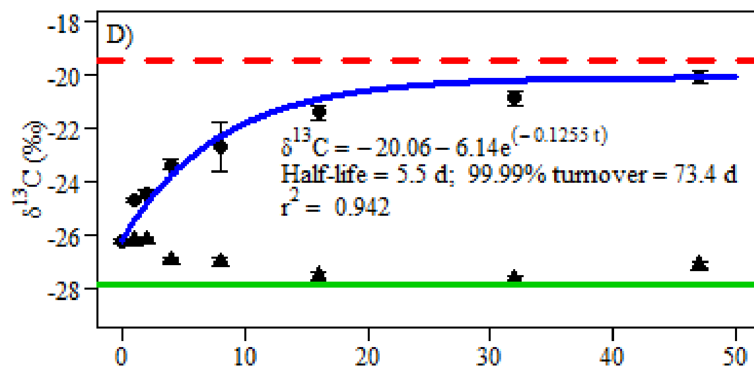
[b]High-protein diet, low-protein diet

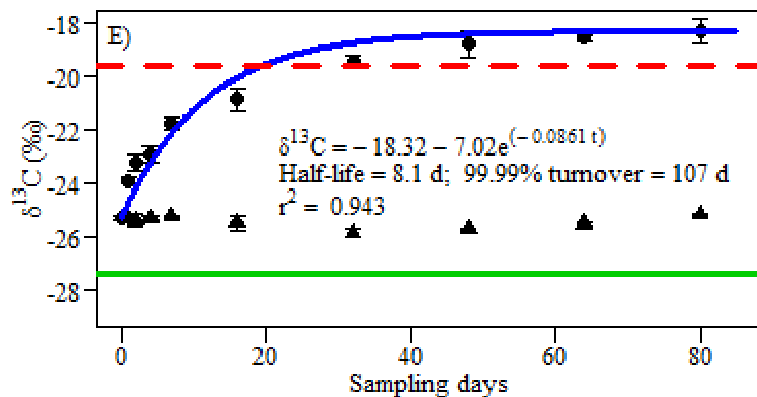
[c]Muscle, gonad


Muscle
A)
$\delta^{13}C = -18.79 - 6.99e^{(-0.1118\,t)}$
Half-life = 6.2 d; 99.99% turnover = 82.4 d
r^2 = 0.993
$\delta^{13}C$ (‰)


Gonads
B)
$\delta^{13}C = -19.1 - 8.03e^{(-0.1738\,t)}$
Half-life = 4 d; 99.99% turnover = 53 d
r^2 = 0.913
$\delta^{13}C$ (‰)


C)
$\delta^{13}C = -20.1 - 4.86e^{(-0.1075\,t)}$
Half-life = 6.4 d; 99.99% turnover = 85.7 d
r^2 = 0.978
$\delta^{13}C$ (‰)


D)
$\delta^{13}C = -20.06 - 6.14e^{(-0.1255\,t)}$
Half-life = 5.5 d; 99.99% turnover = 73.4 d
r^2 = 0.942
$\delta^{13}C$ (‰)


E)
$\delta^{13}C = -18.32 - 7.02e^{(-0.0861\,t)}$
Half-life = 8.1 d; 99.99% turnover = 107 d
r^2 = 0.943
$\delta^{13}C$ (‰)
Sampling days

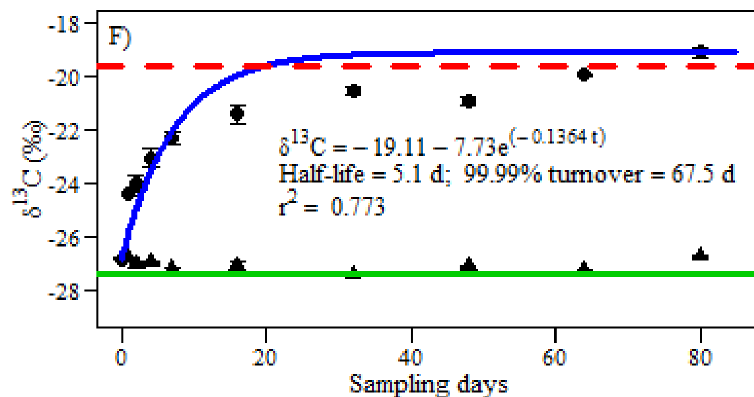

F)
$\delta^{13}C = -19.11 - 7.73e^{(-0.1364\,t)}$
Half-life = 5.1 d; 99.99% turnover = 67.5 d
r^2 = 0.773
$\delta^{13}C$ (‰)
Sampling days

Figure 1.1. Mean $\delta^{13}C$ values of muscle (left) and gonad tissue (right) per sampling day for three species of freshwater snails fed on low-protein (triangles) or high-protein diet (dots). (A–B) *Helisoma trivolvis*, (C–D) *Lymnaea elodes* and (E–F) *Lymnaea stagnalis*. For each graph, the blue curve is the best fit curve. The dotted red line represents the mean $\delta^{13}C$ value of the high-protein diet (n = 2 for A–F), the straight green line represents the mean $\delta^{13}C$ value of the lettuce diet (n = 4 for A–B, 5 for C–D, 3 for E–F).

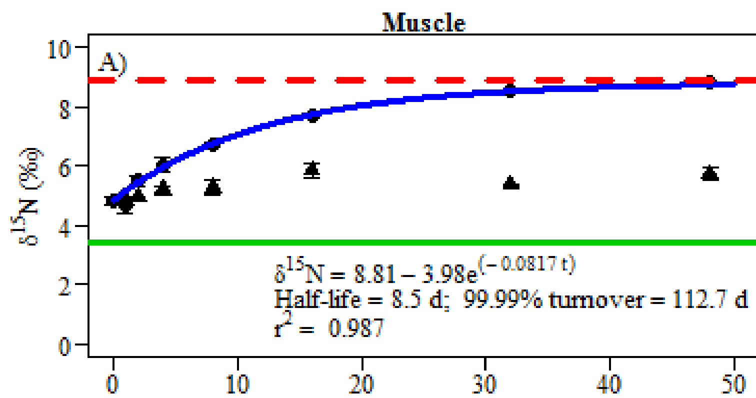
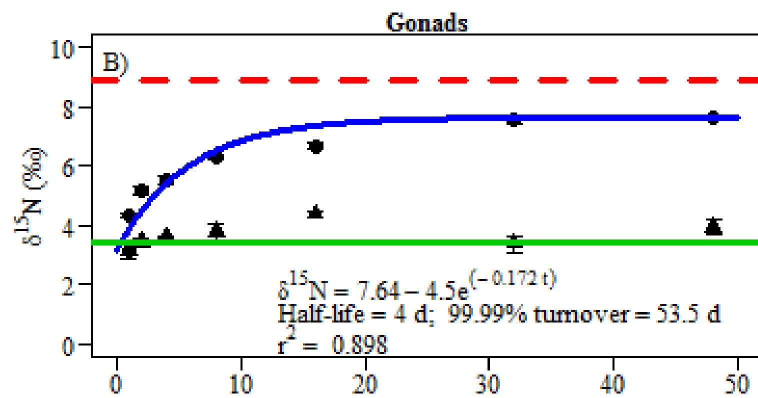
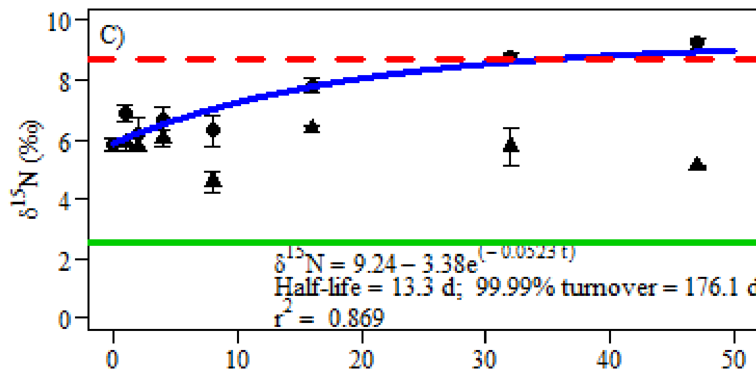
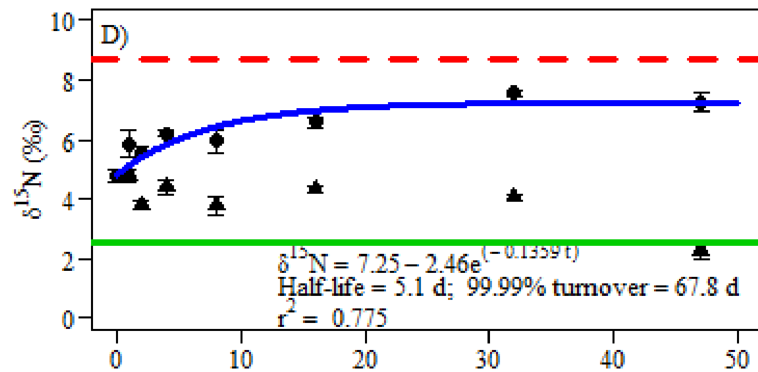
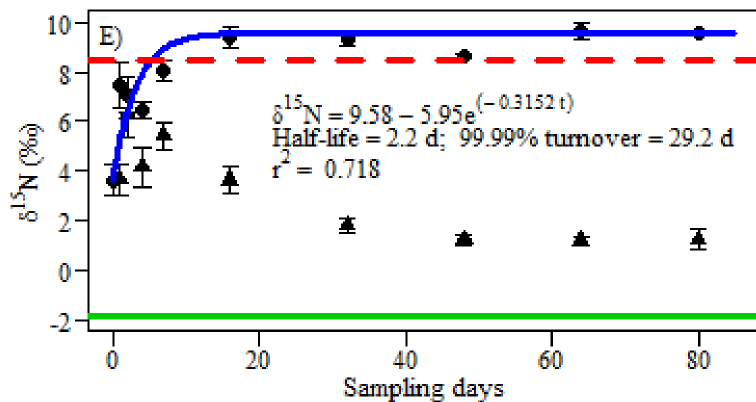
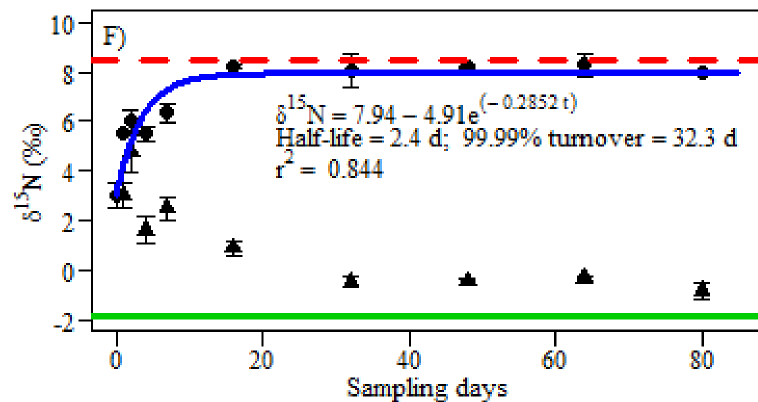

Muscle
A)
$\delta^{15}N = 8.81 - 3.98e^{(-0.0817\,t)}$
Half-life = 8.5 d; 99.99% turnover = 112.7 d
$r^2 = 0.987$
$\delta^{15}N$ (‰)

Gonads
B)
$\delta^{15}N = 7.64 - 4.5e^{(-0.172\,t)}$
Half-life = 4 d; 99.99% turnover = 53.5 d
$r^2 = 0.898$
$\delta^{15}N$ (‰)

C)
$\delta^{15}N = 9.24 - 3.38e^{(-0.0523\,t)}$
Half-life = 13.3 d; 99.99% turnover = 176.1 d
$r^2 = 0.869$
$\delta^{15}N$ (‰)

D)
$\delta^{15}N = 7.25 - 2.46e^{(-0.1359\,t)}$
Half-life = 5.1 d; 99.99% turnover = 67.8 d
$r^2 = 0.775$
$\delta^{15}N$ (‰)

E)
$\delta^{15}N = 9.58 - 5.95e^{(-0.3152\,t)}$
Half-life = 2.2 d; 99.99% turnover = 29.2 d
$r^2 = 0.718$
$\delta^{15}N$ (‰)
Sampling days

F)
$\delta^{15}N = 7.94 - 4.91e^{(-0.2852\,t)}$
Half-life = 2.4 d; 99.99% turnover = 32.3 d
$r^2 = 0.844$
$\delta^{15}N$ (‰)
Sampling days

Figure 1.2. Mean $\delta^{15}N$ values of muscle (left) and gonad tissue (right) per sampling day for three species of freshwater snails fed on low-protein (triangles) or high-protein diet (dots). (A–B) *Helisoma trivolvis*, (C–D) *Lymnaea elodes* and (E–F) *Lymnaea stagnalis*. For each graph, the blue curve is the best fit curve. The dotted red line represents the mean $\delta^{15}N$ value of the high-protein diet (n = 2 for A–F), the straight green line represents the mean $\delta^{15}N$ value of the lettuce diet (n = 4 for A–B, 5 for C–D, 3 for E–F).

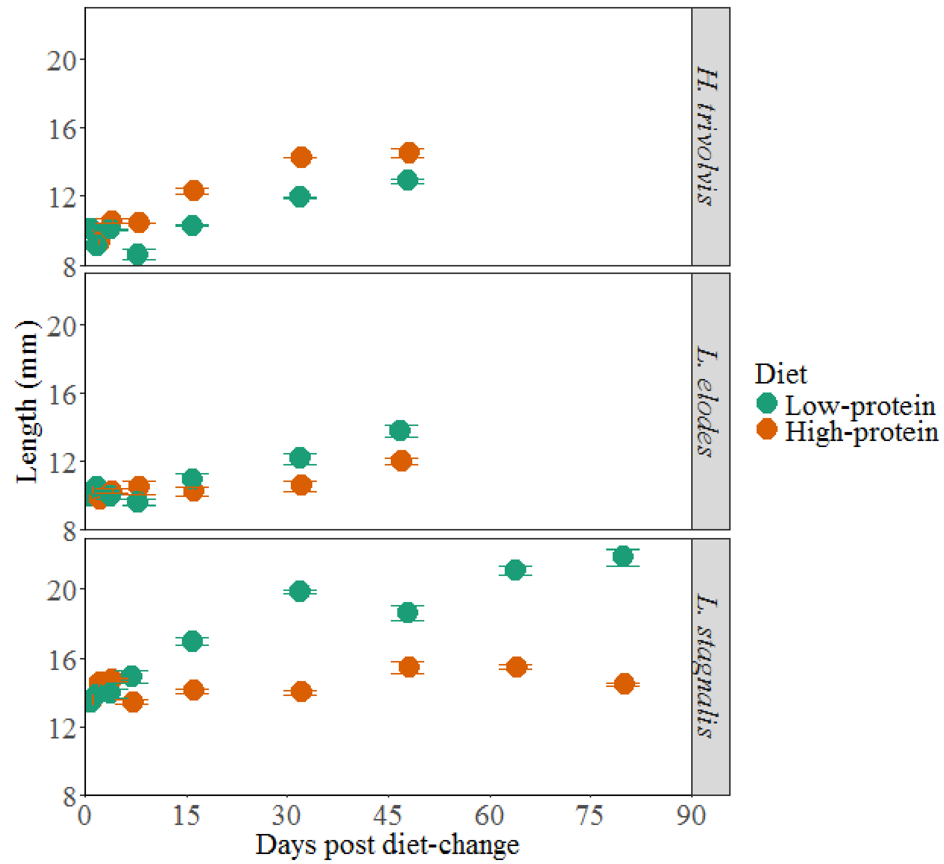


Figure 1.3. Shell length (mean ± SE, mm) of three freshwater snail species fed a low-protein and high-protein diet.

Chapter 2: Quantifying incorporation rates of nutrition sources to better estimate concentration dependence for stable isotope mixing models

Introduction

Stable isotope analysis is increasingly being used to reconstruct the diets of wildlife because the relative contribution of different food sources can be estimated (Friesen and Roth, 2016; Hopkins and Kurle, 2016). For this analysis, stable isotope abundance from elements like carbon and nitrogen is measured from the consumer tissue and its potential/known food sources. Then these values are incorporated into Bayesian stable isotope mixing models (Franco-Trecu *et al.*, 2013; Parnell *et al.*, 2013). The accuracy of results from these models depends in part on concentration dependence and diet-tissue discrimination factors. For many species and tissues, both these parameters are unknown and specifying different values can lead to different estimates of the proportion of particular plant or animal food items in diets (Ben-David and Schell, 2001; Phillips and Koch, 2002; Bond and Diamond, 2011). Thus, it is important that these two parameters are calculated for more species, and the factors that influence them are better understood.

Concentration dependence takes into account the concentration of elements in each food item and is important to use when there is a large difference in the concentration of elements (e.g., [C] and [N]) among the food items in a diet. Mixing models without concentration dependence assume that each food source contributes equal amounts of carbon and nitrogen to a consumer's tissue. However, food sources (e.g. plants compared to animals) can have distinct element concentrations. For omnivorous animals, it is important to incorporate concentration dependence when reconstructing the diet with stable isotope analysis because they may eat a mixture of plants with lower nitrogen concentrations (higher C:N) and animal prey with

relatively higher nitrogen concentrations (low C:N) (Phillips and Koch, 2002; Phillips *et al.*, 2014). Failure to account for the variation in element concentration among food items can bias estimates of diet (Koch and Phillips, 2002; Phillips and Koch, 2002). For example, the estimated proportion of salmon in brown bear's diet varied from 59% to 26% between models with and without concentration dependence (Phillips and Koch, 2002).

In early studies, concentration dependence was taken from the [C] and [N] that resulted from the measurement of stable isotope ratios (e.g. Phillips and Koch, 2002). However, using these values assumes that all the elements have been equally digested, which may not be the case especially when these items include plant and animal tissue (Robbins *et al.*, 2002). Several studies have shown that digestion rates vary among different dietary macromolecules (carbohydrate, lipid and protein; Robbins, 1993). These studies show that dietary nitrogen mainly stems from protein but dietary carbon comes from different sources including carbohydrate, lipid and protein. The few studies that have accounted for digestibility when calculating concentration dependence found no difference in the concentration of carbon and nitrogen with and without digestibility (Koch and Phillips, 2002). Despite this result, later studies used values that accounted for digestibility because in theory digestibility could matter (Hopkins and Kurle, 2016). To make concentration dependence values even more realistic, differences in the incorporation of elements into tissue during metabolism could be considered. However, few studies have determined incorporation rates, so it has not been possible to understand their influence on concentration dependence in Bayesian stable isotope mixing models.

Dietary nutrition sources can have different incorporation rates because they can be used in different metabolic pathways. Dietary lipids are preferentially routed to synthesize tissue lipids, and can be metabolized for energy if needed (Krueger and Sullivan, 1984; Tieszen and

Fagre, 1993; Phillips and Koch, 2002). Carbohydrates are preferentially oxidized to produce energy or converted to glycogen to store energy for later use (Hobson and Stirling, 1997), and dietary protein goes primarily into tissue synthesis (Tieszen and Fagre, 1993; MacAvoy *et al.*, 2005). Although all three sources can be used for energy, proteins and lipids are more likely to be incorporated into the tissue relative to carbohydrates (Tieszen and Fagre, 1993). For example, if a diet contains mostly carbohydrate and less amino acids (e.g. herbivores), the consumer might synthesize amino acids from keto acids that were derived from carbohydrates for tissue growth. Consequently, dietary carbohydrate would be incorporated into the tissue. On the other hand, if the diet contains abundant protein and lipid but less carbohydrate (e.g. carnivores), the amino acids from the protein and lipid will contribute to tissue growth, while amino acids from the carbohydrates can be metabolized and used for energy and excreted (Krueger and Sullivan, 1984).

Incorporation rates not only vary because of how macromolecules are metabolized, but also because dietary nutrients are routed differently among tissues within an organism. For example, studies with rats and mice show that dietary protein is preferentially routed into certain tissues, like muscle and liver, for protein synthesis (Ambrose and Norr, 1993; Tieszen and Fagre, 1993). In addition to differences in incorporation due to tissues, incorporation rates could vary within and among species because of differences in diet, differences in metabolism, and isotopic routing among tissues. Variable incorporation rates could further explain the variation in another important parameter in stable isotope analysis – diet-tissue discrimination factor (DTDF).

Animal tissues generally have higher isotope ratios than their diet because lighter isotopes move more rapidly through biochemical pathways and thus molecules containing lighter isotopes are more easily used for energy (Peterson and Fry, 1987). Consequently, molecules with

higher isotope ratios are more likely to be incorporated into the tissue. Studies often use 1‰ and 3.4‰ for carbon and nitrogen DTDFs, respectively (Post, 2002). However, negative DTDFs are occasionally observed, especially with carbon stable isotopes. It has been hypothesized that negative values occur when components of the diet (i.e. protein and carbohydrate) have distinct isotopic signatures and are used and routed differently by the consumer (i.e. for energy, storage, tissue synthesis, etc). Several studies have tested this hypothesis using diets with isotopically distinct nutrition sources. For example, MacAvoy *et al.* (2005) fed mice a diet consisting of a mixture of protein at a lower-isotopic value and carbohydrate at a higher-isotopic value. They found the isotopic value of tissues was lower than the diet (average value of different nutrition sources), but higher than the protein source, which indicated that more dietary protein was assimilated into the tissue (Arneson and MacAvoy, 2005; MacAvoy *et al.*, 2005). Although these studies suggest that protein is more likely to be incorporated into tissues, there are few studies examining incorporation rates especially outside of mammal-laboratory systems. Thus, it remains unclear how variable incorporation rates can be among taxa, tissue types, and different quality diets.

Incorporation rates may explain why DTDFs are affected by several factors including diet quality, and tissue type. If dietary macromolecules are used and routed differently among tissues and organisms, then each tissue and organism will have a different isotopic signature. For example, carbon isotopic signatures varied among primarily producers that used different photosynthetic pathways to fix atmospheric CO_2 into their systems, with lower $\delta^{13}C$ values in C_3 than C_4 plants (-26 to -28‰ vs -12 to -14‰; Tieszen, 1991). In addition, protein is generally higher in $\delta^{13}C$ values than carbohydrate from the same source and lipid is depleted in ^{13}C (Krueger and Sullivan, 1984; Tieszen and Fagre, 1993). Because isotope ratios of consumers

reflect the elements incorporated from the nutrition source, DTDFs could vary with different diets, especially when the diet is complex and includes different sources, as well as by tissue type and species.

Although incorporation rates could help estimate and explain concentration dependence and DTDF in stable isotope mixing models, only a limited number of species and diets have been investigated. Therefore, it is unclear what proportion of an element is incorporated into tissues during digestion, compared to the amount that is lost because it was used for energy and excreted as waste. Thus, the objective of this study was to use different combinations of isotopically distinct nutrition sources to determine the proportion of protein and carbohydrate incorporated into tissue versus the proportion used as an energy source. We used two tissue types (muscle and gonad) from two freshwater snail species; *Lymnaea stagnalis* is an herbivore that feeds primarily on macrophytes and algae (Rybak, 2016), and *Helisoma trivolvis* is a generalist feeder (Brown, 1982). Based on their feeding habits, we predicted that *L. stagnalis* would incorporate more carbohydrate into their tissues than *H. trivolvis*, which might incorporate mostly protein. To determine the contribution of dietary nutrition sources on tissue isotopic values, we derived novel equations that improve upon previous approaches (MacAvoy *et al.*, 2005) because their required assumptions are likely to be met with most biological datasets.

Methods

Diet preparation

Different combinations of protein (casein) and carbohydrate (corn starch) were used to make five diets for snails (Table 2.1). In case snails did not survive on the N-depleted corn starch diet, another diet (Diet C_4) was included that mixed corn starch and corn flour to create a

carbohydrate diet with 1% protein. All foods were finely ground and well mixed with a magnetic stir bar and 83 mL of water. The mixture was poured into a tray (0.5 cm depth) and placed in a drying oven at 60 ºC for 12 hours to prevent the growth of bacteria. Two batches of each diet were made to provide sufficient amounts of food for the duration and number of snails involved in the experiment (Table 2.2).

Tissue sampling

A total of 210 snails per species (*L. stagnalis* and *H. trivolvis*) were raised in individual jars from eggs and fed lettuce *ad libitum* until they were ~10 mm in shell length (~10 weeks). Then 30 snails each were randomly assigned to seven different diets for the remainder of the experiment. Individuals of *L. stagnalis* were all fed the first batch of diets and *H. trivolvis* were fed the first batch for all diets as well as the second batch for Diet C, Starch, C4 and Corn flour (Table 2.2). Snail muscle and gonad tissue was sampled at 56 and 70 days post-diet change for stable isotope analysis (based on Chapter 1 results for turnover time), with two replicates per sampling day with up to eight individuals per replicate. Two-sample t-tests determined whether $\delta^{13}C$ values were different between the time points. If there was no difference between the two time points, we inferred that complete turnover had occurred.

Samples were processed for stable isotope analysis following the protocol described in Chapter 1. Lipid extractions were performed on the diet (n = 18; 1st batch: 7 diets x 2 replicates + 2nd batch: 4 diets x 1 replicate) and snail tissue samples (n = 112, 2 species × 2 tissue types × 7 diets × 2 replicates × 2 sampling days). We also prepared diet samples without lipid extraction (n = 18) and performed Mann-Whitney U tests to determine whether the stable isotope signatures differed between extracted and non-extracted diet samples. No difference was found in $\delta^{13}C$ values between lipid extracted and non-lipid extracted diet samples (W = 165.5, P = 0.924),

which suggested that lipids were not a major component of the diet. Thus, the values from each type of preparation were averaged (Table 2.2). The stable isotope ratios of diets shown in Table 2.2 were used to determine the DTDF of snail tissue on each type of the diet.

Measuring snail performance

Given that incorporation rates were predicted to differ among the diets based on feeding habits, we also predicted that snail size and survival would differ among the diets because of differences in feeding habits. For each snail species, size was assessed for all individuals at each sampling point by measuring snail shell length from apex to aperture (Burch, 1982) using digital calipers. Survival status (alive/dead) was assessed each time snails were fed or had their water changed (almost daily).

Using mixing equations to determine the contribution of dietary nutrition sources to tissue isotope signal

Stable isotope mixing equations can be used to determine the contribution of the sources to a mixture (Ben-David and Schell, 2001; Phillips and Gregg, 2001). In our study, there were two sources, protein and carbohydrate, with distinct carbon isotopic values, but only protein is a source of nitrogen (i.e. there is no nitrogen in carbohydrates). Thus, we focused on carbon and using carbon values in mixing equations. The general form of the mixing equation is:

$$\delta^{13}C_{snail} - DTDF = f_{protein}\delta^{13}C_{protein} + (1 - f_{protein})\delta^{13}C_{carbohydrate} \quad (1)$$

Where $f_{protein}$ is the fraction of diet from protein source, $1 - f_{protein}$ is the fraction of diet from carbohydrate source ($f_{carbohydrate}$), and $\delta^{13}C_{protein}$ and $\delta^{13}C_{carbohydrate}$ represent the mean carbon isotopic values from the protein and carbohydrate sources, respectively. DTDF is the diet-tissue discrimination factor, which corrects for the difference of isotopic values between

animal tissue and the diet. Two previous studies used the above equation to quantify the contribution of different dietary nutrition sources (Arneson and MacAvoy, 2005; Arneson *et al.*, 2006). These studies assumed that DTDFs were the same between two different diets because the nutritional components were identical. In other words, there was a similar proportion of dietary macromolecules (protein, carbohydrate, and lipid) in the diets. They also assumed that all carbon from the diet (from both carbohydrate and protein) was mixed first and then incorporated into the tissue in the same proportions (i.e., dietary carbon was incorporated in proportion to the dietary concentration). However, these studies found that carbon in the tissue mainly came from protein (about 75%), which invalidated their last assumption.

We modified Equation 1 so that it does not require the assumption that DTDFs are the same among diets. Equation 2 allows the proportional contribution of protein and carbohydrate to differ. To determine the contribution of dietary nutrition sources, we used the tissue isotopic values from snails fed with pure protein and pure carbohydrate diet (Diet Casein and Starch) to determine the relative contribution of protein and carbohydrate in the three mixed diets (Diet A, B and C) (Table 2.1).

Snails in our study were fed with different diets including a pure protein (casein) diet and a pure carbohydrate (corn starch) diet. If turnover is achieved, then the isotopic value of the consumer's tissue should reflect the diet they assimilated: $\delta^{13}C_{incorporated\ diet(X)} = \delta^{13}C_{tissue(diet\ X)}$, where $\delta^{13}C_{incorporated\ diet(X)}$ represents the isotopic value of the diet incorporated into the tissue and $\delta^{13}C_{tissue\ (diet\ X)}$ is the mean carbon isotopic value of the tissue from the consumer fed with diet X. Thus, $\delta^{13}C_{incorporated\ casein} = \delta^{13}C_{tissue(casein)}$, and $\delta^{13}C_{incorporated\ starch} = \delta^{13}C_{tissue(starch)}$ for snails fed with pure casein and corn starch,

respectively. For diets that include a mix of protein and carbohydrate, the isotopic value of the incorporated diet should come from incorporated protein and incorporated carbohydrate:

$$\delta^{13}C_{tissue\ (diet\ X)} = f_{protein\ (Diet\ X)}\delta^{13}C_{incorporated\ protein} + (1 - f_{protein\ (Diet\ X)})\delta^{13}C_{incorporated\ carbohydrate} \qquad (2)$$

where $f_{protein\ (Diet\ X)}$ is the fraction of incorporated diet from the protein source and X = A, B, or C.

Similar to previous studies of incorporation rates, some assumptions were necessary. The first assumption was that lipids were not an important component of the diet and that if they were incorporated they were preferentially routed to synthesize more lipids. As such lipids were not a concern in our study because we extracted lipids from all consumer tissues and diet samples to minimize their effect. The second is that $\delta^{13}C_{incorporated\ protein}$ was constant and independent from $f_{protein}$ among diets (Diet Casein, A, B, C and Starch). To date, this assumption has not been tested.

Mixing equations for incorporation rates of nutrition sources

To quantify incorporation rates of nutrition sources in animals, we also designed a novel set of equations that stems from a common equation used in stable isotope studies where the isotopic value of animal tissue reflects the diet with DTDFs:

$$\delta^{13}C_{tissue\ (diet\ X)} = \delta^{13}C_{diet\ X} + DTDF_{diet\ X} \qquad (3)$$

where $\delta^{13}C_{tissue\ (diet\ X)}$ is the carbon isotopic value of the tissue from the consumer fed with diet X. $DTDF_{diet\ X}$ represents the DTDF of the consumer's tissue from diet X. Our first diet was casein, a pure protein source. With Diet Casein, Equation 3 becomes:

$$\delta^{13}C_{tissue(casein)} = \delta^{13}C_{casein} + DTDF_{casein} \qquad (4)$$

This equation describes that consumers incorporate a proportion of casein into the tissue, and the remainder is used as energy and lost as waste. To represent this, the proportion of protein incorporated into the tissue is $P_{protein}$, and the proportion of protein lost is $(1 - P_{protein})$. Because lighter isotopes are more easily excreted, the isotopic value of incorporated protein and not incorporated protein (used as energy and lost as waste) could be different. We made them $\delta^{13}C_{incorporated\ casein}$and $\delta^{13}C_{wasted\ casein}$, respectively. The sum of these two parts should equal the casein diet:

$$\delta^{13}C_{casein} = P_{protein}\delta^{13}C_{incorporated\ casein} + (1 - P_{protein})\delta^{13}C_{wasted\ casein} \quad (5)$$

If turnover is achieved, then the isotopic value of the consumer's tissue should reflect the diet they assimilated. In the case of Equation 5, $\delta^{13}C_{incorporated\ casein} = \delta^{13}C_{tissue(casein)}$.

Equations 4 and 5 can be modified to estimate similar unknown parameters when a consumer is fed a pure carbohydrate diet. In this case, the proportion of carbohydrate incorporated into the tissue is $P_{carbohydrate}$ and the proportion of carbohydrate lost as waste is $(1 - P_{carbohydrate})$. We used $\delta^{13}C_{incorporated\ starch}$ and $\delta^{13}C_{wasted\ starch}$ to represent the isotopic ratios of carbohydrate incorporated into the tissue and lost as waste, respectively. Thus, an equation similar to Equation 5 can be used for a pure carbohydrate diet (Diet Starch):

$$\delta^{13}C_{starch} = P_{carbohydrate}\delta^{13}C_{incorporated\ starch} + \left(1 - P_{carbohydrate}\right)\delta^{13}C_{wasted\ starch} \quad (6)$$

After compete turnover, the isotopic value of consumer tissue should equal that of the incorporated carbohydrate, so $\delta^{13}C_{snail\ (starch)} = \delta^{13}C_{incorporated\ starch}$.

For diets that include a mix of protein and carbohydrate, the proportions of each nutrient source are multiplied by the $\delta^{13}C$ of each protein and carbohydrate source to determine the

overall $\delta^{13}C$ of that diet. For example, in our study Diet A contains 5/6 casein and 1/6 carbohydrate, thus we have:

$$\delta^{13}C_{Diet\ A} = \frac{5}{6}\delta^{13}C_{casein} + \frac{1}{6}\delta^{13}C_{starch} \qquad (7)$$

Substituting Equations 5 and 6 into Equation 7 and rearranging terms gives:

$$\delta^{13}C_{Diet\ A} = \frac{5}{6}P_{protein}\delta^{13}C_{incoporated\ casein} + \frac{5}{6}(1 - P_{protein})\delta^{13}C_{wasted\ casein} + \frac{1}{6}P_{carbohydrate}\delta^{13}C_{incoporated\ starch} + \frac{1}{6}\left(1 - P_{carbohydrate}\right)\delta^{13}C_{wasted\ starch} \qquad (8)$$

In Equation 8, there are two parts accounting for the assimilated diet, which are from incorporated protein ($\frac{5}{6}P_{protein}\delta^{13}C_{incoporated\ casein}$) and incorporated carbohydrate ($\frac{1}{6}P_{carbohydrate}\delta^{13}C_{incoporated\ starch}$). $\frac{5}{6}P_{protein}$ is the proportion of the diet incorporated into the tissue from protein, and $\frac{1}{6}P_{carbohydrate}$ is the proportion of the diet incorporated into the tissue from carbohydrate. The isotopic value of the consumer's tissue should be the sum of the two parts with the proportion:

$$\delta^{13}C_{tissue\ (Diet\ A)} = \frac{\frac{5}{6}P_{protein}}{\frac{5}{6}P_{protein}+\frac{1}{6}P_{carbohydrate}}\delta^{13}C_{incoporated\ casein} + \frac{\frac{1}{6}P_{carbohydrate}}{\frac{5}{6}P_{protein}+\frac{1}{6}P_{carbohydrate}}\delta^{13}C_{incoporated\ starch} \qquad (9)$$

where $\frac{\frac{5}{6}P_{protein}}{\frac{5}{6}P_{protein}+\frac{1}{6}P_{carbohydrate}}$ is the proportion of the incorporated protein in Diet A from both incorporated protein and carbohydrate; $\frac{\frac{1}{6}P_{carbohydrate}}{\frac{5}{6}P_{protein}+\frac{1}{6}P_{carbohydrate}}$ is the proportion of the incorporated carbohydrate in Diet A from both incorporated protein and carbohydrate.

The above equation (Equation 9) can be written as a general form for all the diets (Diet Casein, A, B, C and Starch) as:

$\delta^{13}C_{tissue\ (Diet\ X)} =$

$$\frac{\frac{protein\ mass}{protein\ mass+carbohydrate\ mass}P_{protein}}{\frac{protein\ mass}{protein\ mass+carbohydrate\ mass}P_{protein}+\frac{carbohydrate\ mass}{protein\ mass+carbohydrate\ mass}P_{carbohydrate}}\delta^{13}C_{incorporated\ casein} +$$

$$\frac{\frac{carbohydrate\ mass}{protein\ mass+carbohydrate\ mass}P_{carbohydrate}}{\frac{protein\ mass}{protein\ mass+carbohydrate\ mass}P_{protein}+\frac{carbohydrate\ mass}{protein\ mass+carbohydrate\ mass}P_{carbohydrate}}\delta^{13}C_{incorporated\ starch}$$

(10)

For the diets with pure protein (Diet Casein) and carbohydrate (Diet Starch), $\frac{protein\ mass}{protein\ mass+carbohydrate\ mass}$ and $\frac{carbohydrate\ mass}{protein\ mass+carbohydrate\ mass}$ is either 0 or 1. Thus, from the equation we find that $\delta^{13}C_{tissue\ (casein)} = \delta^{13}C_{incorporated\ casein}$ and $\delta^{13}C_{tissue\ (starch)} = \delta^{13}C_{incorporated\ starch}$, respectively. Using the $\delta^{13}C_{incorporated\ casein}$ and $\delta^{13}C_{incorporated\ starch}$ values gained from Diets Casein and Starch, there are two unknowns ($P_{protein}$ and $P_{carbohydrate}$) for three equations with Diet A, B and C, respectively.

This set of equations uses the same two assumptions already discussed (see *Using mixing equations to determine the contribution of dietary nutrition sources to tissue isotope signal*). In addition, these equations assume that the proportion of protein ($P_{protein}$) and carbohydrate ($P_{carbohydrate}$) incorporated into the tissue was constant among diets.

Results

There was no difference in δ^{13}C values between 56 and 70 days post-diet change (t = -0.64, df = 110, *P* = 0.523) indicating that snails completed their turnover. Consequently, average values from the two time points were used in the mixing equations.

Because turnover was completed, we determined the carbon DTDFs with different diets in this study. As predicted, snails fed diets with higher concentrations of the C_4 carbohydrate

source had lower $\Delta^{13}C$ values and negative values were observed as the proportion of protein decreased in the diet (Diets B, C, Starch, C_4 and Corn flour) from Diet A (Figure 2.1).

Table 2.2 shows the results from the mixing equations that were used to determine the contribution of protein ($f_{protein}$) and carbohydrate ($1 - f_{protein}$, $f_{carbohydrate}$) in newly synthesized tissue. Overall, the main nutrition source contributing to tissue synthesis varied by the proportion of the nutrition sources in the diet. Diet A contained more protein than carbohydrate, and protein contributed relatively more to the isotopic value of the snail tissue ($f_{protein}$ = 63–83%). In contrast, Diet C had more carbohydrate than protein, and the model results showed that carbon was mainly incorporated from carbohydrates ($f_{carbohydrate}$ = 59–96%). In Diet B where the proportion of nutrition sources was the same, $f_{protein}$ and $f_{carbohydrate}$ were similar for *H. trivolvis*; however, *L. stagnalis* incorporated more carbohydrate than protein.

Using the mixing equations that were developed to determine incorporation rates of dietary nutrition sources, we were unable to calculate the exact $P_{protein}$ and $P_{carbohydrate}$. Ratios of $P_{protein}$ to $P_{carbohydrate}$ were calculated for each of the mixed diets (Diet A, B and C), but the ratios were different for each diet. For instance, with *H. trivolvis* muscle tissue, the ratio of $P_{protein}$ to $P_{carbohydrate}$ was 0.71, 1.08 and 3.47 with Diet A, B and C, respectively (Table 2.2). With three different ratios of $P_{protein}$ to $P_{carbohydrate}$ the absolute values of $P_{protein}$ and $P_{carbohydrate}$ cannot be calculated using our equations. This result demonstrates that one of the assumptions for our equations, constant incorporation of the proportion of protein and carbohydrate no matter the diet, was violated. Thus, we reported the ratio of $P_{protein}$ to $P_{carbohydrate}$ for each of the mixed diets (Diet A, B and C) for each tissue type and each snail

species (Table 2.2). This ratio indicates which dietary nutrition source (protein or carbohydrate) had a higher incorporation rate in a particular diet. A ratio > 1 suggested protein had a higher incorporation rates, < 1 meant that carbohydrate had higher incorporation rates, and = 1 indicated that protein and carbohydrate had the same incorporation rates. In general, *L. stagnalis* had higher incorporation rates of carbohydrate than protein, especially for muscle tissue. For *H. trivolvis*, the source with higher incorporation rates depended upon the diet source concentrations: diet with less protein tended to have higher protein incorporation rates than carbohydrate.

Diet had an effect on snail survival and growth, but species had different responses (Table 2.4 and Figure 2.2). *Helisoma trivolvis*, which is considered a generalist feeder, had high survival no matter the diet (93–100% survival). However, *L. stagnalis* had comparatively lower survival rates (53–80%). In terms of growth, *L. stagnalis* grew larger when fed extreme diets consisting of pure protein (Diet Casein) or pure carbohydrate (Diet Starch) compared to diets with 50% protein (Diet B) and 17% protein (Diet C). Mean lengths on the extreme diets were 20.64 ± 2.43 mm and 19.75 ± 1.02 mm, while mean lengths on Diet B and C were 16.68 ± 0.12 mm, and 16.65 ± 0.48 mm, respectively. In contrast, *H. trivolvis* grew the largest on Diet C (13.94 ± 0.22 mm), and was smallest (7.58 ± 0.12 mm) when fed pure carbohydrate (Diet Starch).

Discussion

We demonstrated that carbon DTDFs vary with the proportion of protein in the diet. Variation in DTDFs could be related to our finding that the contribution of protein and carbohydrates to tissues also varied by diet. In more protein-rich diets, protein contributed most

to the isotopic value in the tissue (63–83%) relative to carbohydrates regardless of feeding habit. When the diet consisted mainly of carbohydrates (Diet C), the herbivorous species *L. stagnalis* rarely incorporated protein (e.g., 4% for muscle). However, on the same diet about 40% of the tissue was derived from dietary protein for the generalist feeding species *H. trivolvis*. We were unable to calculate incorporation rates with our equations, though these equations demonstrated that the proportion of protein and carbohydrate incorporated into the tissue was not constant among diets. Furthermore, we found that the proportion of protein in the diet affected snail growth and survival according to feeding habits.

By comparing the $\delta^{13}C$ of the diet and snail tissues, we generated carbon DTDFs for two freshwater snails on different diets. We observed mostly negative DTDFs and only found positive DTDFs with the two diets highest in protein (Figure 2.1). DTDFs are normally positive because lighter isotopes move more rapidly through biochemical pathways and heavier isotopes remain in tissue for tissue synthesis (Peterson and Fry, 1987). Negative DTDFs were first observed in Diet B (half protein and half carbohydrate), and these values became lower as the concentration of the C_4 carbohydrate source increased. These negative DTDFs support the assumption that components of the diet with different isotopic signatures were being assimilated differently. Therefore, when estimating DTDFs with controlled feeding studies, researchers should use diets with proportions of C_3 and C_4 sources that are similar to the food items consumed in nature.

By using different combinations of diets, we quantified the proportion of newly synthesized tissue derived from protein and carbohydrate. Previous studies have attempted to quantify this using one diet that was assumed to have a constant DTDF with nutrition sources in the same proportion but consisting of different isotopic values. This assumption might be invalid

because diet isotopic values can influence DTDF (see review by Caut *et al.*, 2009). The novelty of Equation 2 is that it does not require that assumption and is able to test whether and how concentration of a nutrition source in the diet influences the contribution of the source to tissue synthesis. Our results showed that protein does not always contribute more than carbohydrate to tissue isotopic values, as suggested by previous studies (MacAvoy *et al.*, 2005; Arneson *et al.*, 2006). In our study, the contribution of the dietary nutrition source to tissue synthesis depended on the concentration of the source in the diet and the feeding habits of the organism (Table 2.3).

The contribution of the dietary nutrition source to the tissue isotopic value increased as the proportion of that source in the diet increased (Table 2.3). For example, when a diet consisted of more protein (83%) and less carbohydrate (17%) (Diet A), carbon in the tissue was mainly derived from dietary protein (63-83%); whereas when the diet had more carbohydrate (83%) and less protein (17%) (Diet C), protein only contributed 4–41% of the carbon in tissue. The diet used by MacAvoy *et al.* (2005) contained approximately 21% protein and 59% carbohydrate, which is similar to our Diet C. However, they suggested that 75% of carbon was incorporated into the tissue from protein, which is the opposite from our results (Table 2.3). These contrasting results could be due to the fact that MacAvoy et al. (2005) violated two important assumptions: 1) constant DTDF with nutrition sources in the same proportion but consisting of different isotopic values (discussed above); 2) all carbon from dietary components was incorporated into the tissue in the same proportion. Another potential reason for differences between the studies is that the organisms being used were taxonomically quite distinct (rodent vs gastropod), and as a result might have different needs for certain dietary nutrition components. More taxa need to be studied to understand to what degree generalities can be made and how protein and carbohydrates contribute to tissue synthesis.

By performing diet studies with two species of gastropods, we demonstrated that the contribution of a certain nutrition source to tissue synthesis differed between species. *Lymnaea stagnalis* incorporated less protein and more carbohydrate into their tissues than *H. trivolvis.* Especially with Diet C (contained mostly carbohydrate), protein only contributed to 4% of the muscle and 21% of the gonad tissue's isotopic value in *L. stagnalis.* In contrast, protein contributed to 41% of the muscle and 39% of the gonad tissue's isotope signal in *H. trivolvis* consuming Diet C. These results supported our prediction that species with different feeding habits would differ in how nutrition sources contributed to tissue synthesis. The herbivore, *L. stagnalis*, feeds primarily on macrophytes in nature (Rybak, 2016), whereas *H. trivolvis* is a generalist feeder (Brown, 1982) that grew faster on diets with more protein than carbohydrate (Li, Chapter 1).

Given the difference in feeding habits, it was not surprising to find that species differed in their growth and survival on different diets. As the proportion of protein decreased from 100–17%, *H. trivolvis* increased in size to reach its maximum size on Diet C (17% protein). However, shell length then decreased as the proportion of protein decreased (7–0%); the smallest snails observed were fed pure carbohydrate. These results were consistent with results from Chapter 1, which found that growth performance was best when *H. trivolvis* had some protein in the diet. Our current study indicates that peak growth performance occurs when the diet contains approximately 17% protein. *Helisoma trivolvis* had high survival no matter the diet, suggesting that variation in food quality may affect size, but does not strongly affect survival in nature.

In contrast, the largest *L. stagnalis* were fed two extreme diets that consisted of either 100% protein (Diet Casein) or 0% protein (Diet Starch). To further contrast *H. trivolvis*, this species was smallest when fed Diet B and Diet C. Although growth was similar on these two

diets, survival rates for *L. stagnalis* were highest on Diet B (80%), and lowest (53%) on Diet C. When comparing survival and growth among all diets, there is some evidence for a trade-off between growth and survival. When fed 50% protein:carbohydrate (Diet B), *L. stagnalis* had high survival but low growth whereas on extreme diets (pure carbohydrate or pure protein), survival rates decreased but those who survived grew more.

Using the equations to determine incorporation rates of the dietary nutrition source, we were not able to calculate the exact $P_{protein}$ and $P_{carbohydrate}$ because $P_{protein}$ and $P_{carbohydrate}$ was not constant among the diets. However, we were able to calculate a ratio of $P_{protein}$ to $P_{carbohydrate}$, revealing which incorporation rates were higher compared with the other. We found that this ratio was influenced by diet and species. *Lymnaea stagnalis* had higher carbohydrate incorporation rates than that of protein except for the gonad tissue with Diet C. Compared to *L. stagnalis*, *H. trivolvis* tended to have higher incorporation rates of protein than carbohydrate. With Diet C, both muscle and gonad tissue for *H. trivolvis* had protein incorporation rates that were three times higher than that of carbohydrate.

This is the first study to use a series of diets to test how the proportion of protein:carbohydrate affects the contribution of dietary nutrition sources on newly synthesized tissue. Our study showed that the contribution of the dietary nutrition source to tissue synthesis tissue was positively related to the concentration of the source in the diet and influenced by feeding habits. Previous studies only suggested the role of protein in tissue synthesis, whereas our study demonstrated that carbohydrate can also be an important source for tissue isotope signal when diets consist of a higher proportion of carbohydrate or primarily herbivorous species are examined. Controlled-feeding studies of incorporation rates such as our study may inform more than just ecological stable isotope studies. Ecological stoichiometry, which measures

differences in the concentrations and ratios of elements (e.g., C, N, P) between consumers and their diet, may benefit from a better understanding of where consumers derive energy and how they allocate it to different functions such as tissue synthesis. By quantifying the contribution of dietary nutrition sources (i.e., protein, carbohydrate) to consumer tissue synthesis with different diets, we can suggest the underlying mechanisms on how consumers maintain a constant ratio of elements (e.g., C:N) when eating different diets with a wide range of C:N ratios.

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Table 2.1. The components and proportion of protein within each of the seven diets fed to freshwater snails.

Diet	Casein (g)	Corn starch (g)	Corn flour (g)	Proportion of protein in the diet (%)
Casein	60	0	0	100
A	50	10	0	83
B	30	30	0	50
C	10	50	0	17
Starch	0	60	0	0
C_4	0	51.3	8.7	1
Corn flour	0	0	60	7

Table 2.2. The mean stable carbon isotope ratios (± SD) of the seven diets fed to freshwater snails. Some snails were fed from a combination of batch 1 and 2 depending upon the diet and species.

Diet	Species[a]	Batch	n[b]	$\delta^{13}C$ ± SD (‰)
Casein	1, 2	1	4	-27.38 ± 0.02
A	1, 2	1	4	-25.31 ± 0.30
B	1, 2	1	4	-20.26 ± 0.78
C	1	1	4	-14.02 ± 0.46
C	2	1, 2	4, 2	-13.76 ± 0.54
Starch	1	1	4	-10.88 ± 0.01
Starch	2	1, 2	4, 2	-10.88 ± 0.02
C_4	1	1	4	-11.00 ± 0.08
C_4	2	1, 2	4, 2	-10.99 ± 0.06
Corn flour	1	1	4	-11.65 ± 0.12
Corn flour	2	1, 2	4, 2	-11.64 ± 0.10

[a] 1–*Lymnaea stagnalis*; 2–*Helisoma trivolvis*

[b] Number of samples from each batch processed for stable isotope analysis

Table 2.3. The mean stable carbon isotope ratios (± SD), the contribution of protein ($f_{protein}$) and carbohydrate ($1 - f_{protein}$, $f_{carbohydrate}$) in newly synthesized tissue, and the incorporation rate ratio of dietary protein to carbohydrate ($P_{protein}/P_{carbohydrate}$) for muscle (M) and gonad (G) from freshwater snails *Lymnaea stagnalis* and *Helisoma trivolvis*. Snails were fed seven diets that varied in the proportion of protein.

Species	Tissue type	Diets	N	$\delta^{13}C$ ± SD (‰)	$f_{protein}$ (%)	$f_{carbohydrate}$ (%)	$P_{protein}/P_{carbohydrate}$
L. stagnalis	M	Casein	4	-25.03 ± 0.11	100	0	-
L. stagnalis	M	A	4	-22.58 ± 1.03	63	37	0.34
L. stagnalis	M	B	4	-20.52 ± 1.22	31	69	0.45
L. stagnalis	M	C	4	-18.75 ± 1.15	4	96	0.21
L. stagnalis	M	Starch	4	-18.47 ± 0.62	0	100	-
L. stagnalis	M	C_4	4	-18.36 ± 1.14	-	-	-
L. stagnalis	M	Corn flour	4	-17.00 ± 0.45	-	-	-
L. stagnalis	G	Casein	4	-25.89 ± 0.41	100	0	-
L. stagnalis	G	A	4	-23.86 ± 0.40	81	19	0.85
L. stagnalis	G	B	4	-20.06 ± 1.37	44	56	0.79

L. stagnalis	G	C	4	-17.68 ± 0.92	21	79	1.33
L. stagnalis	G	Starch	4	-15.44 ± 1.02	0	100	-
L. stagnalis	G	C_4	4	-15.99 ± 0.41	-	-	-
L. stagnalis	G	Corn flour	4	-15.54 ± 0.60	-	-	-
H. trivolvis	M	Casein	4	-24.71 ± 0.21	100	0	-
H. trivolvis	M	A	4	-23.12 ± 0.25	78	22	0.71
H. trivolvis	M	B	4	-21.20 ± 0.24	52	48	1.08
H. trivolvis	M	C	4	-20.40 ± 0.10	41	59	3.47
H. trivolvis	M	Starch	4	-17.36 ± 0.55	0	100	-
H. trivolvis	M	C_4	4	-13.11 ± 0.76	-	-	-
H. trivolvis	M	Corn flour	4	-11.80 ± 0.51	-	-	-
H. trivolvis	G	Casein	4	-25.98 ± 0.12	100	0	-
H. trivolvis	G	A	4	-23.76 ± 0.26	83	17	0.98
H. trivolvis	G	B	4	-19.36 ± 0.45	48	52	0.82
H. trivolvis	G	C	4	-18.16 ± 0.53	39	61	3.20
H. trivolvis	G	Starch	4	-13.16 ± 0.67	0	100	-

H. trivolvis	G	C_4	4	-11.77 ± 0.14	-	-	-
H. trivolvis	G	Corn flour	4	-11.43 ± 0.24	-	-	-

Table 2.4. Survival rates of *Lymnaea stagnalis* and *Helisoma trivolvis* on seven different diets in the experiment.

	Survival rates (%)	
Diets	*L. stagnalis*	*H. trivolvis*
Casein	60	93
A	73	93
B	80	100
C	53	100
Starch	60	100
C_4	67	100
Corn flour	60	100

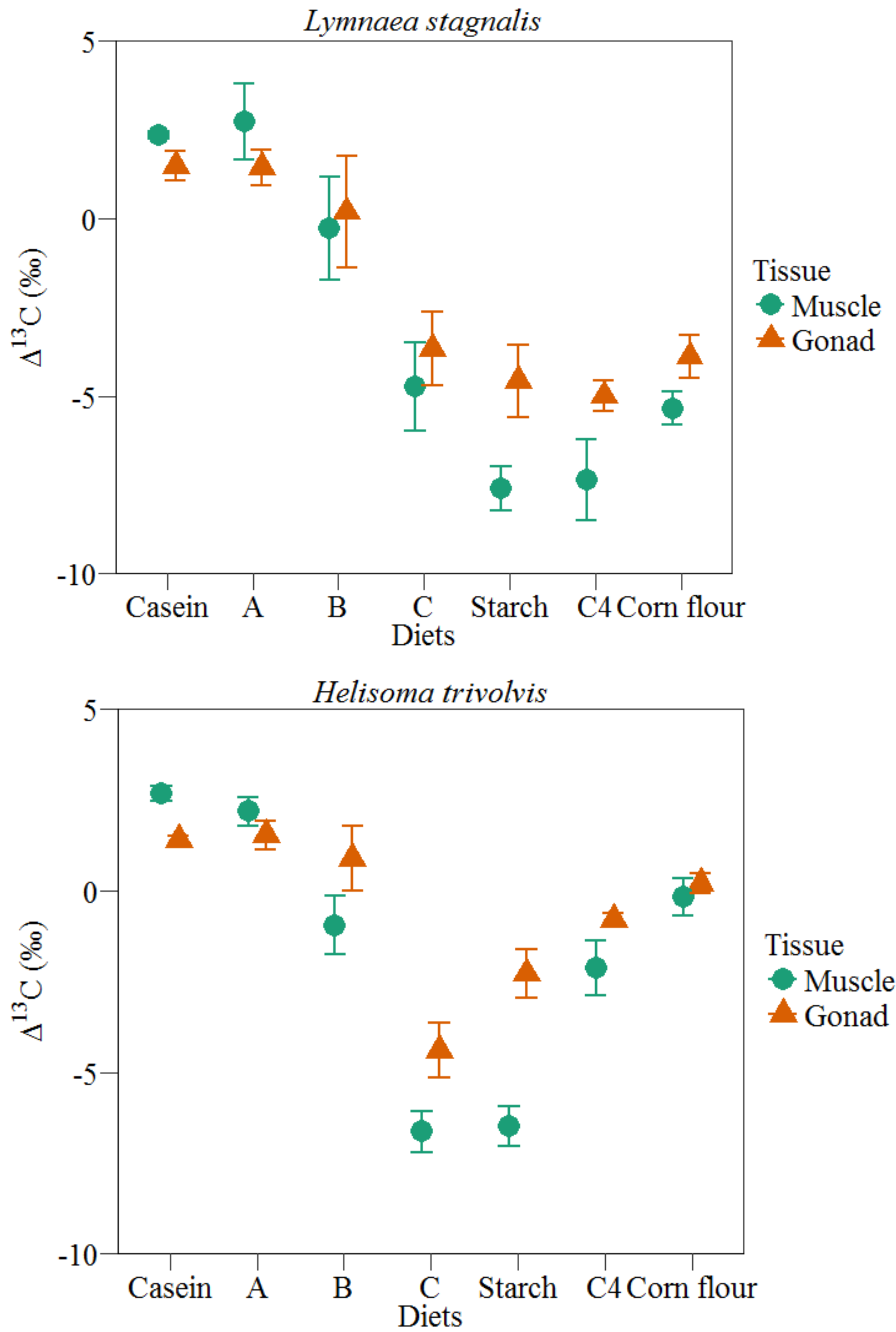

Figure 2.1. Carbon diet-tissue discrimination factors ($\Delta^{13}C$, mean ± SD) for muscle and gonad tissue of freshwater snails *Lymnaea stagnalis* and *Helisoma trivolvis* fed on seven diets.

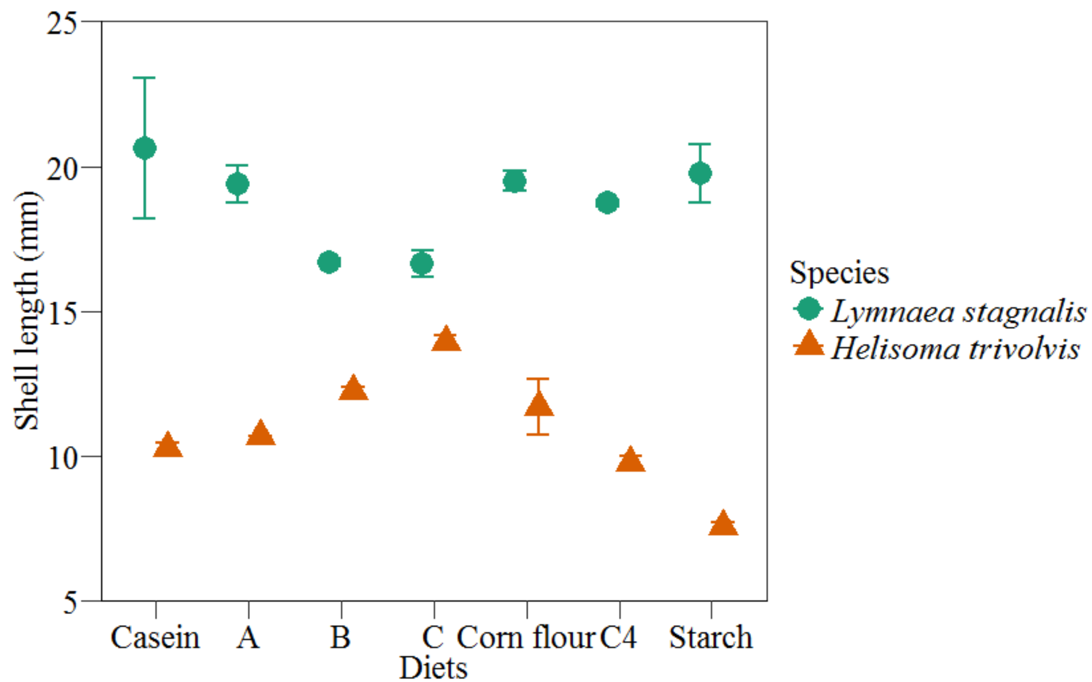

Figure 2.2. Mean shell length (± SD) of freshwater snails *Lymnaea stagnalis* and *Helisoma trivolvis* fed on seven different diets. Diets appear in order of decreasing protein concentration.

Chapter 3: Stable isotope analysis indicates host-parasite encounter rates and reveals unexpected routes of parasite transmission

Introduction

Estimates of parasite transmission are important because they can be used to predict disease dynamics. However, it is often unclear how realistic the outcomes are due to a lack of empirical data associated with the parameters in the models. One parameter that is included in almost all models of transmission is "encounter" (i.e. β in Anderson and May, 1979). For many trophically-transmitted parasites, hosts encounter parasites via the consumption of food items. Yet, wildlife diet is often unknown or difficult to quantify, so it can be difficult to measure encounter rates in the field, and thus generate good parameters for theoretical models.

A widely used tool to reconstruct a consumer's diet is stable isotope analysis, which assumes that stable isotope ratios in animal tissues reflect their diet. Stable isotope ratios (e.g. carbon and nitrogen) are measured in the consumer's tissue and from potential food sources (plant and animal tissue). These values are then used into isotopic mixing models that are computed with Bayesian statistics to determine the relative contribution of each food source in a consumer's tissue (Parnell *et al.*, 2010). In other words, stable isotope analysis quantifies the proportions of particular food sources in a consumer's diet. Although whole animals can be used, this tool is of practical use because often a sample of muscle or hair is enough to indicate the diet (Peterson and Fry, 1987). Further, this approach is especially useful for diet analysis of omnivorous animals which have a complex diet that otherwise can be difficult to resolve.

The accuracy and reliability of stable isotope analysis depends upon several assumptions including that all the potential plant and prey items in the diet are sampled (Gannes *et al.*, 1997). To test the latter assumption, diet could be independently assessed from parasite infection

patterns. Trophically-transmitted parasites, such as many trematodes, serve as indicators of diet because their presence within a host is a record of even a very brief visit to a particular location (Johnson and McKenzie, 2009). Hosts become infected when they consume trematodes that are encysted on a plant, or when intermediate hosts (e.g. snails, fish, frogs, or arthropods) with internal cysts are consumed. However, using parasites to infer diet may not be as useful when life cycle information is not known or inaccurate (Bolek *et al.*, 2016). Further, many parasites have complex life cycles (use several hosts to complete their life cycles) and are not strictly host specific (use a variety of hosts at a given point in their life cycle). For example, some trematodes like *Echinostoma trivolvis* can infect several different second intermediate hosts like freshwater snails and tadpoles, and so the presence of these parasites would not clearly indicate which prey item was consumed.

The objective of our study was to determine if integrating diet estimates from stable isotope analysis and trematode parasite infection patterns improves our understanding of wildlife diet, parasite life cycles and parasite transmission. We used a muskrat-trematode system because muskrats are thought to be primarily herbivorous, but occasionally eat animal prey including crayfish, catfish (Errington, 1963), and mussels (Stearns and Goodwin, 1941). They are commonly hosts to several trematode species that have either a three- or two-host life cycle (McKenzie and Welch, 1979; Detwiler *et al.*, 2012). In muskrats, the three-host life cycle parasites always use a mollusc as a first intermediate host. Several larval stages develop and asexually reproduce within the first host, eventually producing a swimming stage (cercaria) that emerges from the snail. Cercariae must be consumed or penetrate second intermediate hosts, which could be molluscs, insects, and amphibians. For example, second intermediate hosts for *E. trivolvis* includes freshwater snails, *Plagiorchis noblei* infects aquatic insects, and *Plagiorchis*

proximus infects aquatic insects and freshwater snails (McMullen, 1937; Blankespoor, 1971). Within the second intermediate hosts, the parasites migrate and then encyst within the tissues. When these hosts are consumed, the parasites excyst and develop into adults within the intestinal tract of the muskrat. For two-host life cycle parasites, trematode larvae emerge from first intermediate host molluscs and encyst on plants that infect definitive hosts when plants are consumed. For muskrats, infections with *Quinqueserialis quinqueserialis* indicate plant consumption.

Because both methods of inferring diet have drawbacks, we integrated them and used the identified parasite species to determine what food items to sample for stable isotope analysis, and used the stable isotope analysis to infer whether previous knowledge of parasite life cycles and transmission was accurate. We hypothesized that if diet is related to encounter rates, then diet estimates for food items involved in parasite life cycles will be positively related to parasite infection patterns. Examining the relationship between the proportion of a food item in a consumer's diet and the abundance and intensity of their parasites could confirm parasite life cycles, reveal new routes of transmission, and help calibrate encounter rates in models of parasite transmission.

Methods

Parasite and tissue sampling from hosts

Muskrats from three wetlands within southern Manitoba (N = 67) were collected by fur trappers during Fall 2014 and Spring 2015 (locations not listed to comply with fur trappers' wishes). Frozen, usually skinned carcasses of muskrats were necropsied and species diversity, prevalence, mean abundance and infection intensity of trematodes was assessed. Samples of

muscle tissue (two 1.5 mL vials) were collected from muskrats (left thigh) were preserved at -20 °C for stable isotope analysis. Consumer tissue was processed for stable isotope analysis following the protocol described in Chapter 1.

Prey and plant sampling

Within each trapping area, prey and plants were sampled because they could be transmitted by the parasites recovered from the muskrats (Table 3.1). Up to five species of aquatic plants (*Equisetum fluviatile*, *Typha latifolia*, *Phragmites* spp., *Carex* spp., and *Schoenoplectus tabernaemontani*) were sampled because *Q. quinqueserialis* could encyst on these plants. In addition, previous studies suggested that muskrats eat these plants and they should be found at most sites in our study (Takos, 1947; Errington, 1948; Danell, 1978; Jelinski, 1989; Lacki *et al.*, 1990; Kiviat, 2013). Animal prey that could transmit 3 species of trematodes were sampled including freshwater snails (*Lymnaea elodes*, *Helisoma trivolvis*, *Lymnaea stagnalis*, *Physa gyrina* and *Promenetus* spp.), fish (*Pimephales promelas*, *Esox lucius*, *Culaea inconstans*, *Ameiurus nebulosus*, *Notropis volucellus* and *Pimephales notatus*), insect larvae (*Corydalus* spp., *Sympetrum* spp., *Aeshna* spp.) and tadpoles (*Lithobates sylvaticus*) were twice sampled within the trapping area of the three wetlands in 2016 (Spring = May, Fall = August). For stable isotope analysis, fish (tail), tadpole (tail) and snail (foot) and frozen at. The specific region for each animal was selected to reduce the chances that parasite-infected tissue was used, as signatures from parasites themselves may influence stable isotope ratios (Arneson *et al.*, 2006). Due to their small size, the whole body of insect larvae was preserved at -20 °C for stable isotope analysis.

Statistical analysis

We tested the assumption that food sources were isotopically distinct with a MANOVA, which is required for concentration-dependent MixSIAR modeling (Stock and Semmens, 2013). Isotopic values of $\delta^{13}C$ and $\delta^{15}N$ from individuals associated with each of the four food item groups (plant, snails, insects and vertebrates) were determined (Table S.1). MANOVA analysis indicated that within each muskrat group, the isotopic values of $\delta^{13}C$ and $\delta^{15}N$ differed among the food item groups (Marsh Fall: $F_{3,4} = 8.78$, $P = 0.031$; Marsh Spring: $F_{3,6} = 16.22$, $P = 0.003$; Lake Fall: $F_{3,6} = 6.63$, $P = 0.025$; River Spring: $F_{3,6} = 4.39$, $P = 0.059$). Because tadpoles were only collected in spring at the Marsh site, and fish were only collected in the other three muskrat groups, these two prey sources were grouped together as vertebrates for the MixSIAR analysis. In total, the MixSIAR models used the mean isotopic values of $\delta^{13}C$ and $\delta^{15}N$ from the four food item groups to estimate the proportion of each food item group (Table 3.2).

Concentration dependence was necessary to include in the MixSIAR models because plant and animal prey contained different concentrations of carbon and nitrogen. We ran models with two sets of concentration dependence to determine how these different sets of values would affect the diet estimates. The first set of concentration dependence parameters (CD1) accounted for digestibility. The percent of water, protein, fat, carbohydrate, and ash in each food item by weight was found in the USDA Nutrient Database (www.nal.usda.gov/fnic/foodcomp/search). Then these weight percentages were used to calculate the overall concentration of carbon and nitrogen digested from each food item following Hopkins and Kurle (2016) (Appendix A: Table A.3). To account for differences in assimilation rates, we calculated another set of concentration dependence parameters (CD2) (Appendix A: Table A.4) based on Robbins *et al.* (2002).

In addition to concentration dependence, we specified values for diet-tissue discrimination factors (DTDFs) in our models to make the stable isotope data for consumers and

their diet comparable, because consumers generally have higher carbon and nitrogen stable isotopic values than their prey due to lighter isotopes being more easily excreted. To our knowledge, no study has determined DTDF for muskrats. The only study of muskrat diet using stable isotope ratios (Hersey *et al.*, 2013) applied DTDFs based on the average carbon and nitrogen values from different taxa including shrimp, insects, rats and mice (DeNiro and Epstein, 1978; Minagawa and Wada, 1984). Then, those values were applied for both plant and animal prey in Hersey *et al.* (2013). However, previous studies show that DTDFs vary by species and diet quality (Robbins *et al.*, 2005; Steinitz *et al.*, 2016). Thus, we ran models with two sets of DTDF values because it was unclear which species and diet would best fit wild muskrats. DTDF1 was generated from laboratory mice (*Mus musculus*; Arneson and MacAvoy, 2005) and DTDF 2 came from laboratory rats (*Rattus norvegicus*; Kurle *et al.*, 2014). In both studies, consumers were fed known diets composed of either pure plants (wheat) or animal matter (casein or fish meal). Because these studies found different DTDFs depending upon the composition of the diet, we applied different values for plant and animal prey in our study (Table 3.3).

In addition to specifying concentration dependence and DTDFs, our MixSIAR models used uninformative priors for all food sources because although muskrats are hypothesized to eat mainly plants, there was no prior information on the proportion of plants or different prey items in the diet. We used 50 000 iterations with burn-in of 5000 and a thinning rate of *15, resulting in a posterior distribution composed of 3000 estimates of proportional source contributions. Our analysis estimated the diet for four muskrat groups (based on location and season; Table 3.2) as well as for individual muskrats in three of these groups. No individual diet estimates for the Fall Marsh muskrat group were conducted because the stable isotope ratios of plants and insects were indistinguishable, and concentration-dependent MixSIAR modeling requires that all food sources

have isotopically distinct values. Group estimates and individual estimates were run with process*residual error and process error only, respectively. Fall food sources (collected in mid-August) were used for muskrats collected in Fall (late October) because the turnover time for muscle tissue from rats and mice was 60 days (Miller *et al.*, 2008; Kurle, 2009). For Spring muskrats (collected in late April or May), we ran stable isotope mixing models with Spring food sources (May) because we did not collect plants or prey in February due to the logistical difficulties of sampling under ice. Based on personal observations at each site, we assumed that abiotic conditions/biotic communities in May were more similar to February compared to August.

With groups of muskrats, four MixSIAR models with two sets of diet-tissue discrimination factor values and two sets of concentration dependence values were run. The results from the models with DTDF1 and CD2 were reported herein for several reasons. First, DTDF1 was reported because the $\Delta^{15}N$ of plants was higher than that of animals. This outcome fits an assumption that the $\Delta^{15}N$ increases as protein quality decreases in animal diet (Robbins *et al.*, 2005) and the results from turnover studies (Chapter 1). Second, results with CD2 were reported because digestibility and assimilation rates were accounted for. The results from the remainder of the models can be found in Appendix B. For each MixSIAR model's output, we reported both mean and mode proportional estimates of four food items (one plant and three prey) in the diet (Stock and Semmens, 2013). Regression analysis was used to test the relationship between the proportion of four food sources (mode) and trematode parasitism. Mean abundance and intensity of infection of each parasite species was used for the analysis determining the relationship of food sources to muskrat groups (4 food sources x 4 parasite species x 3 groups = 48 regression analyses). Intensity of infection of each parasite species was

regressed against the proportion of each food source for individuals in three muskrat groups (4 food sources x 4 parasite species x 3 groups = 48 analyses). We performed linear regression using R statistical software v3.2.5 (R Core Team, 2016). Data were log-transformed if the assumptions of normality and homogeneity of variance were not met.

Results

Four species of trematodes infected muskrats from our sampling sites (Table 3.4). *Quinqueserialis quinqueserialis* was the most prevalent parasite (59–82%) from all three sites and both seasons. Muskrats collected from the lake in Fall (Lake Fall group) had the highest abundance and intensity of infection with *P. noblei*, and muskrats from the river (River Spring group) had the highest abundance and intensity of infection with *P. proximus* and *E. trivolvis*.

Stable isotope ratios of muskrats and their prey varied both spatially and temporally (Figure 3.1). From the final models, diet estimates showed that muskrats from all groups primarily ate plants (Figure 3.2; Table 3.5: mean: 67.9–89.8%, mode: 79.1–94.9%). For the prey items, snails contributed the most to the diet of the River Spring group (Figure 3.2; Table 3.5: mean = 8.2%, mode = 8.7%) and Lake Fall group (Figure 3.2; Table 3.5: mean = 6.7%, mode = 4.3%). Vertebrates (frog and fish) accounted for more of the diet in the Lake Fall group (Figure 3.2; Table 3.5: mean = 10.9%, mode = 8.9%) and Marsh Spring group (Figure 3.2; Table 3.5: mean = 9.6%, mode = 9.3%).

The relationship between diet and parasite abundance and mean intensity among muskrat groups was not significant for any parasite species ($P > 0.05$). For example, the abundance and mean intensity of the plant-encysting parasite, *Q. quinqueserialis*, was not significantly related to the proportion of plants in the diet, respectively ($F_{1,2} = 0.002$, $P = 0.969$, $r^2 = -0.50$, Figure 3.3A;

$F_{1,2}$ =0.44, P = 0.439, r^2 = -0.03, Figure 3.3B). There was no relationship between the proportion of vertebrates in the diet and the mean abundance of *E. trivolvis* ($F_{1,2}$ =0.56, P = 0.531, r^2 = -0.17). However, in some cases a positive trend may emerge if more muskrat groups were sampled. Our results suggest that additional muskrat groups could be assessed to better understand the relationship between the proportion of snails in the diet and the abundances of parasites that encyst within second intermediate host snails: *E. trivolvis* ($F_{1,2}$ =5.14, P = 0.152, r^2 = 0.58) (Figure 3.3C) and *P. proximus* ($F_{1,2}$ =4.97, P = 0.155, r^2 = 0.57) (Figure 3.3D).

Individuals within groups varied in the proportion of the food items they ate. However, at all three sites in which individual diets were estimated, all muskrats ate more plants than the highest ranked prey item. In the Lake Fall group, the mode proportion of plants and vertebrates ranged from 56–95% and 1–10% among individuals (Table 3.6). In the Marsh Spring group, individuals ate more plants than vertebrates; the mode proportion ranged from 78–94% and 1–10%, respectively (Table 3.7). For the River Spring group, the mode proportion of plants and snails ranged from 75–99% and 1–22% among individuals (Table 3.8). The proportions for the other food items accounted for less of the diet (mode < 0.02), thus they were not included in the tables.

Regression analysis focused on the relationship between the proportion of plants in individual diets and the plant-encysting parasite *Q. quinqueseralis* because its prevalence was highest among sites. As predicted, there was a significantly positive relationship between percent of plant in the diet and *Q. quinqueseralis* infection from Lake individuals ($F_{1,15}$ = 3.87, P = 0.041, r^2 = 0.20, Figure 3.4A). However, no relationship was found for individual muskrats at the Marsh ($F_{1,8}$ = 0.38, P = 0.556, r^2 = -0.07, Figure 3.4B). In contrast, there was a trend toward a negative relationship between the proportion of plants and *Q. quinqueseralis* in the River

muskrats ($F_{1,39} = 3.90$, $P = 0.062$, $r^2 = 0.12$, Figure 3.4C). In light of this negative relationship, we tested the relationship between the top ranked prey item and parasitism at the River site. Intriguingly, as the proportion of snails in the diet increased, there was significantly more *Q. quinqueseralis* infection ($F_{1,21} = 5.16$, $P = 0.034$, $r^2 = 0.16$, Figure 3.4D).

Discussion

This study showed that parasite infection provides information about the diet of wildlife. The plant-encysting trematode *Q. quinqueserialis* was the most common parasite in muskrat groups and individuals, which supports the conclusions of other studies and the isotope analysis from this study that muskrats are primarily herbivorous. Parasite infection patterns also suggested that muskrats ate freshwater snails or tadpoles because of infection with *E. trivolvis*. Both these prey items are rarely mentioned as important food items for muskrats, but stable isotope analysis also suggested the presence of these prey items in the diet. Thus, identifying parasites and knowing their life cycles helped to inform the prey sampling for stable isotope analysis. Among muskrat groups, there were no significant relationships between the proportion of food items and parasitism, but in several cases there were trends that suggested that if more sites were sampled a significant relationship may be found. Individual estimates showed diet variation among muskrats within groups, and confirmed predicted as well as unpredicted relationships to parasitism. Thus, individual estimates were important in confirming parasite life cycles and revealing new routes of transmission.

The most common parasite recovered in this study was the plant-encysting trematode *Q. quinqueserialis*, which occurred in 70% of muskrats, with up to 325 worms per infection. This result was consistent with other muskrat parasite surveys in North America (MacKinnon and

Burt, 1978; McKenzie and Welch, 1979), where *Q. quinqueserialis* was the most abundant parasite with prevalence as high as 93%. The other three species recovered in muskrats were *E. trivolvis*, *P. noblei* and *P. proximus*, which are all animal-encysting parasites. They were commonly observed across sites but varied in their mean abundance and mean intensity among the four groups of muskrats in this study (Table 3.4). The highest abundance and intensity of *E. trivolvis* and *P. proximus* was observed in muskrats from the river (River Spring group) and the highest abundance and intensity of infection of *P. noblei* was observed in muskrats collected from the lake in Fall (Lake Fall group). These parasites used snails and aquatic insect larvae as second intermediate hosts before being transmitted to definitive hosts (McMullen, 1937; Blankespoor, 1971; Detwiler *et al.*, 2010), which suggested that snails and insect larvae can be potential food items for muskrats. Although other studies of muskrat diet have not reported these sources as prey items, our study demonstrates that identifying parasites and knowing their life cycles can help ensure that potential food items are included in stable isotope analysis.

Our study suggests that it is important to consider the habitat and season in which the consumers and food sources are collected for stable isotope analysis. Most stable isotope studies only sample food items for their consumer once (e.g. Hersey *et al.*, 2013) and sometimes food samples are not collected from the same habitat as the consumer (Friesen and Roth, 2016). Our study showed that the location of the prey sampling relative to the consumer sampling influences the diet estimate because the stable isotope signature of food items varied by location (even just in southern Manitoba area) and time of the year (Spring vs Fall). This might explain why the isotopic signatures of muskrats from different sites and different seasons within the Marsh site clustered together on the isotopic plot (Figure 3.1). Thus, sample time and location is important in diet studies using stable isotope analysis.

The muskrat diet reconstruction results showed that muskrats from all groups primarily ate plants, which was consistent with the consensus from previous studies that muskrats are primarily herbivores that occasionally consume animal matter (Willner *et al.*, 1980). Approximately 87% of the diet was plant, which suggests that animal prey accounted for about 13% of the diet. Although it is unclear whether prey were eaten by accident or intentionally, we suggest that this proportion is high enough to indicate that animal prey were not ingested solely by accident.

Positive trends were found between the proportion of snails in the diet and *E. trivolvis* and *P. proximus* mean abundance from diet estimates of muskrat groups. However, these relationships were not significant probably because only four groups of muskrats were included in this study. Future studies could test if a significant relationship would occur if more sites were sampled. Another potential reason for the lack of a relationship stems from the fact that parasites almost always fit a negative binomial distribution. In other words, most of the prey or plants are infected by a small numbers of parasites, and only a few hosts or substrates are highly infected (Shaw *et al.*, 1998). Muskrats could become infected by eating a few, highly parasitized prey or plants, thus the proportion of a food item in the diet does not directly translate into high infection especially for individual muskrats.

With the individual muskrat diet estimate results, the relationship between diet and parasitism varied by location indicating that different parasite transmission patterns occurred in different habitats. At the River site, there was a slightly negative relationship between the proportion of plants in the diet and plant-encysting parasite intensity, but a significantly positive relationship was found between the proportion of snails in the diet and the intensity of the plant-encysting parasite. The encystment patterns of this parasite are not well described. In nature,

cercariae emerge from the snail and then encyst on vegetation. However, in the laboratory they encyst on the surfaces of containers in 3–5 minutes (Herber, 1942). In laboratory, we observed these parasites encysting on snail shells. Likewise, regression analyses suggest that at the river site, *Q. quinqueserialis* is more likely transmitted by snails than plants, which is a previously unrecognized route of transmission for this parasite.

Overall, this study showed that by combining stable isotope analysis and parasite infection data, we gained more insights into the food items that should be sampled for diet reconstruction, and on parasite transmission patterns. Based on parasite infections and life cycles, we identified potential prey items for muskrats that were not mentioned in other studies such as freshwater snails, tadpoles, and insects. Including these prey items and other potential food items for muskrats in stable isotope mixing models confirmed and quantified the degree to which muskrats eat plants, and animal prey items that are part of muskrat parasite life cycles. Future studies could incorporate the proportions of the diet for each food item into models of parasite transmission to quantify how often hosts encounter a potential source of infection. The regression results suggest that "encounter" would also have to include the probability of parasitism for each food item (e.g. prevalence of infection), as strong relationships were usually not observed between the proportion of a food item in the diet and parasitism. However, if "encounter" included the probability that a food item was consumed along with an estimate reflecting the chance that the food item was parasitized, then future models may better approximate parasite transmission in nature. For instance, if comparisons between diet and parasitism show that lightly infected hosts are consuming a high proportion of infected prey then this suggests that perhaps excystation success is low. In contrast, other factors may explain why hosts can become highly infected when the diet consists of a low proportion of infected prey. In

other words, bringing in quantitative information about infection and not just the presence/absence of a prey item allows for more in-depth inferences about factors mediating parasitism. By integrating stable isotope analysis and patterns of parasitism, we discovered a new route of transmission for the plant-encysting parasite *Q. quinqueserialis*. For many parasites, life cycles have not been studied at all, or have been studied from a limited perspective (Bolek *et al.*, 2016). Most life cycle studies are completed in one part of the parasite's distribution, or in a limited context (usually only field studies rather than in the laboratory), leading to potential misconceptions about the hosts involved in transmission. By using stable isotope analysis, we could address the gaps in our knowledge about wildlife diet and parasite life cycles which could help us understand which routes of transmission have the strongest influence on wildlife disease.

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Table 3.1. Potential intermediate hosts of trematode parasites recovered from muskrats suggests what prey and plant items to sample to reconstruct muskrat diet with stable isotope ratios.

Trematodes	1st intermediate hosts	2nd intermediate hosts
Echinostoma trivolvis	Freshwater snails	Freshwater snails and tadpoles[a]
Plagiorchis noblei	Freshwater snails	Aquatic insect larvae and naiads (midges, damselflies and dragonflies)[b]
Plagiorchis proximus	Freshwater snails	Aquatic insects larvae (e.g. dragonflies, mosquitoes), and sometimes tadpoles, snails and small fishes[c]
Quinqueserialis quinqueserialis	Freshwater snails	None. After the cercariae emerge from the snail they readilty encyst on vegetation and form metacercariae[d]

[a] **Detwiler, J. T., Minchella, D.J.** (2009) Intermediate host availability masks the strength of experimentally-derived colonisation patterns in echinostome trematodes. International Journal for Parasitology 39:585-590.

[b] **Blankespoor, H. D.** (1971). Host-parasite relationships of an avian trematode, *Plagiorchis noblei* Park 1936. Dissertation Abstracts International 31B, 7682.

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[d] **Herber, E. C.** (1942). Life history studies on two trematodes of the subfamily Notocotylinae. The Journal of Parasitology 28, 179-194.

Table 3.2. Mean stable isotope ratios (± SE) of food items for muskrats collected from three sites in southern Manitoba in Spring 2015 or Fall 2014.

Muskrat Group	Food items	n	$\delta^{13}C \pm SE$	$\delta^{15}N \pm SE$
Marsh Spring	Vertebrates	5	-31.00 ± 0.08	5.48 ± 0.12
	Insects	4	-31.57 ± 0.02	10.85 ± 0.30
	Plants	5	-26.85 ± 0.51	5.12 ± 0.72
	Snails	6	-31.80 ± 0.28	9.72 ± 0.40
Marsh Fall	Vertebrates	3	-33.81 ± 0.28	10.53 ± 1.29
	Insects	3	-27.85 ± 0.08	9.02 ± 0.12
	Plants	3	-27.41 ± 0.40	7.00 ± 1.06
	Snails	4	-32.04 ± 0.62	3.03 ± 1.10
Lake Fall	Vertebrates	5	-27.94 ± 0.15	7.35 ± 0.44
	Insects	5	-28.98 ± 0.23	4.21 ± 0.73
	Plants	3	-27.25 ± 0.38	2.54 ± 1.05
	Snails	9	-28.61 ± 0.35	3.15 ± 0.50
River Spring	Vertebrates	2	-29.73 ± 0.08	18.55 ± 0.12
	Insects	3	-31.16 ± 0.08	14.82 ± 0.12
	Plants	4	-27.78 ± 0.40	10.76 ± 1.37
	Snails	14	-32.15 ± 0.38	11.90 ± 0.99

Table 3.3. Diet-tissue discrimination factors for carbon ($\Delta^{13}C$) and nitrogen ($\Delta^{15}N$) from controlled feeding studies of mice and rats. The diet-tissue discrimination factors from wheat diet were used for plant sources in the current study. For animal sources, $\Delta^{13}C$ values were from mice or rats fed with animal protein (casein or fish meal) with a C_3 energy source to minimize the difference between $\delta^{13}C$ value of protein and carbohydrate in the diet; $\Delta^{15}N$ values were from mice or rats fed with fish meal with a C_4 energy source because the $\delta^{15}N$ value of the diet was more similar to the values of prey from our study.

		$\Delta^{13}C$		$\Delta^{15}N$	
Study	Diet	Mean	SD	Mean	SD
Mus musculus	Wheat	1.2	0.3	3.1	0.2
(Arneson, 2005)	Casein (with C_3 energy source)	1.5	0.4	-	-
	Fish meal (with C_4 energy source)	-	-	2.0	0.5
Rattus norvegicus	Wheat	1.40	0.2	2.25	0.1
(Kurle *et al.*, 2014)	Fish meal (with C_3 energy source)	2.05	0.4	-	-
	Fish meal (with C_4 energy source)	-	-	3.05	0.2

Table 3.4. Trematode parasite species diversity, abundance, and mean intensity in muskrats collected from three sites in southern Manitoba in Spring 2015 or Fall 2014.

Muskrat Group	Parasite Species	$N_{Parasite}$[a]	Abundance (± SE)	Intensity (± SE)
Lake Fall	*Quinqueserialis quinqueserialis*	14	30.7 ± 19.0	37.3 ± 22.9
N = 17	*Echinostoma trivolvis*	7	2.2 ± 1.0	5.4 ± 2.0
	Plagiorchis proximus	8	3.4 ± 1.5	7.1 ± 2.8
	Plagiorchis noblei	12	40.5 ± 14.6	57.4 ± 18.8
Marsh Fall	*Quinqueserialis quinqueserialis*	10	29.8 ± 9.4	50.7 ± 12.2
N = 17	*Echinostoma trivolvis*	4	1.1 ± 0.9	4.8 ± 3.4
	Plagiorchis proximus	5	20.4 ± 10.7	69.2 ± 26.6
	Plagiorchis noblei	1	0.1	1.0
Marsh Spring	*Quinqueserialis quinqueserialis*	6	18.2 ± 9.2	30.3 ± 13.4
N = 10	*Echinostoma trivolvis*	2	3.6 ± 2.4	18.0 ± 2.0
	Plagiorchis proximus	2	0.3 ± 0.2	1.5 ± 0.50
	Plagiorchis noblei	0	0.0	0.0
River Spring	*Quinqueserialis quinqueserialis*	17	23.2 ± 8.9	31.4 ± 11.4
N = 23	*Echinostoma trivolvis*	14	7.6 ± 2.7	12.4 ± 4.0
	Plagiorchis proximus	12	71.1 ± 48.5	136.3 ± 90.5
	Plagiorchis noblei	3	6.0 ± 5.9	46.3 ± 44.8

[a] Number of infected hosts

Table 3.5. Dietary contributions (%) (mean, mode and 95% credible interval) for muskrats collected from three different sites in southern Manitoba (Marsh, Lake, and River) in two seasons (Spring 2015, Fall 2014).

Muskrat	Vertebrates			Insect			Plant			Snail		
groups	Mean	Mode	95% CI	Mean	Mode	95% CI	Mean	Mode	95% CI	Mean	Mode	95% CI
Lake Fall	10.9	8.9	1.9–22.5	5.4	0.2	0.2–14.8	77.1	79.1	58.0–91.2	6.7	4.3	1.6–15.7
Marsh Fall	3.0	2.7	0.7–5.8	24.8	1.0	0.0–71.0	67.9	94.9	16.0–97.0	4.2	1.4	0.3–11.1
Marsh Spring	9.6	9.3	2.6–17.2	2.2	0.2	0.1–6.4	85.1	84.7	78.4–91.2	3.1	1.4	0.3–8.3
River Spring	0.9	0.5	0.2–2.4	1.1	0.2	0.0–4.2	89.8	89.6	85.0–94.3	8.2	8.7	3.1–12.9

Table 3.6. Dietary contributions (%) of two food items (mean, mode and 95% credible interval) for individual muskrats collected from the Lake in Fall 2014.

Muskrats	Plant			Vertebrates		
ID	Mean	Mode	95% CI	Mean	Mode	95% CI
1	73.2	75	47.3–91.2	13.8	10	2.8–30.5
2	71.5	89	25.2–97.3	15.4	1	0.6–49.0
3	67.7	87	20.4–96.9	18.5	2	0.7–57.3
4	65.2	79	18.2–96.6	18.1	4	0.8–53.7
5	56.3	56	11.5–95.7	14.4	3	0.9–41.2
6	57	64	10.1–95.0	24.7	5	1.0–71.9
7	56.7	70	11.0–95.4	23.6	1	0.9–69.1
8	72.3	90	26.1–97.1	11.8	1	0.5–39.1
9	63.1	81	11.6–95.7	24.5	3	0.9–79.6
10	56.8	75	10.1–95.6	29.7	1	0.9–75.2
11	78	91	38.5–97.6	10.2	1	0.5–36.0
12	55.8	71	12.0–94.7	19.1	2	1.0–54.0
13	71.4	85	26.7–97.2	14.0	1	0.6–44.2
14	60.2	72	12.3–96.0	26.5	1	1.0–72.2
15	65.4	83	19.3–96.0	15.0	2	0.9–45.5
16	74.1	90	29.7–97.3	12.2	3	0.6–40.5
17	85.7	95	55.4–98.4	9.6	1	0.4–37.0

Table 3.7. Dietary contributions (%) of two food items (mean, mode and 95% credible interval) for individual muskrats from the Marsh in Spring 2015.

Muskrats	Plant			Vertebrates		
ID	Mean	Mode	95% CI	Mean	Mode	95% CI
1	84.3	86	74.2–92.3	8.7	7	1.8–18.3
2	81.6	82	65.9–94.5	11.8	8	0.7–28.3
3	76.1	78	58.6–90.7	12.9	10	0.9–31.8
4	89.1	91	76.8–97.4	6.6	1	0.4–18.5
5	92.3	94	82–98.6	4.8	1	0.3–14.8
6	89.1	92	76.5–98.0	7.7	4	0.4–20.3
7	81.3	81	68.3–92.6	5.8	2	0.5–16.6
8	84	85	71.8–93.9	5.3	2	0.4–16.1
9	89.6	91	78.4–97.4	6.4	2	0.5–17.3
10	90.5	92	78.8–98.4	6.7	1	0.3–18.7

Table 3.8. Dietary contributions (%) (mean, mode and 95% credible interval) for individual muskrats from the River in Spring 2015.

Muskrats	Plant			Snail		
ID	Mean	Mode	95% CI	Mean	Mode	95% CI
1	93.6	94	89.0–97.2	5.2	4	1.8–9.7
2	84.7	83	69.8–97.6	13.1	13	1.3–27.8
3	97.7	99	93.2–99.7	1.6	1	0.1–5.5
4	95.9	98	89.0–99.6	2.8	1	0.2–8.8
5	93.9	97	84.4–99.3	4.4	1	0.3–12.8
6	93.8	96	83.8–99.4	4.7	1	0.3–13.5
7	91.1	94	79.9–98.9	6.8	3	0.5–16.9
8	89.2	89	76.6–98.2	8.3	5	0.8–20.0
9	92.8	93	82.8–99.2	5.5	2	0.4–14.7
10	92.1	96	81.5–99.6	6.3	1	0.4–16.2
11	95.7	98	88.7–91.6	3.2	1	0.2–9.6
12	74.4	75	54.5–99.6	22.3	22	4.6–42.0
13	95.8	99	88.3–91.6	3.2	1	0.2–10.1
14	86.2	88	72.3–99.6	11.4	10	1.0–24.7
15	94.3	97	84.0–97.8	3.5	1	0.3–10.5
16	89.3	90	76.3–99.4	8.3	6	0.8–20.3
17	89.4	92	77.3–98.8	8.7	8	0.6–20.8
18	90.2	92	78.3–98.6	7.4	5	0.6–18.5

19	88.2	92	75–98.5	9.4	8	0.8–21.9
20	90.2	92	78.5–98.8	7.6	7	0.6–18.7
21	82.7	85	66.1–96	13.1	11	1.7–28.1
22	81.4	83	64.7–95.6	14.6	14	2.0–31.3
23	93.6	96	83.1–99.3	4.6	1	0.3–13.2

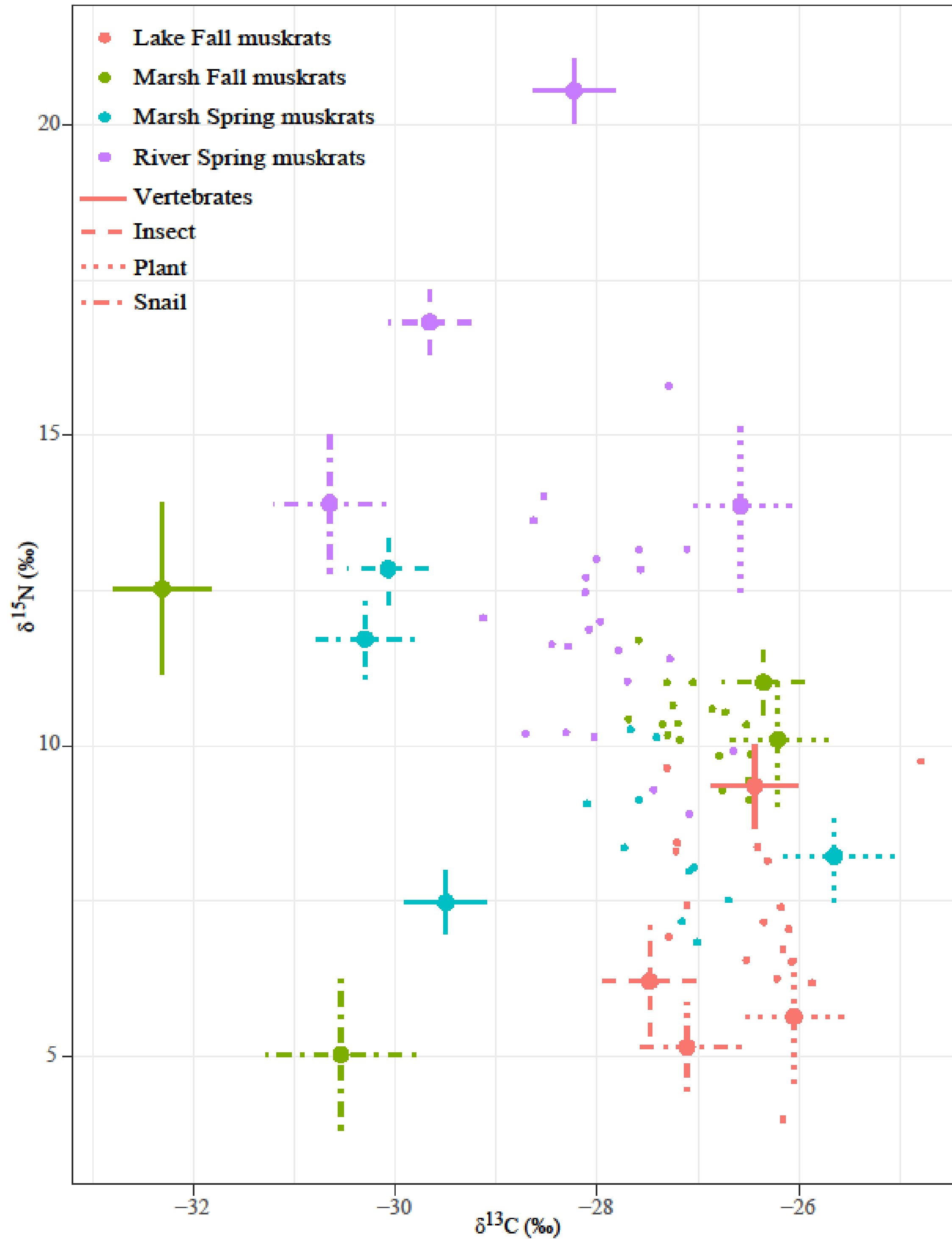


Figure 3.1. Stable isotope plot of muskrats and prey items from four groups from southern Manitoba. Prey means were adjusted using diet-tissue discrimination factor from Arneson and MacAvoy, 2005 (DTDF1).

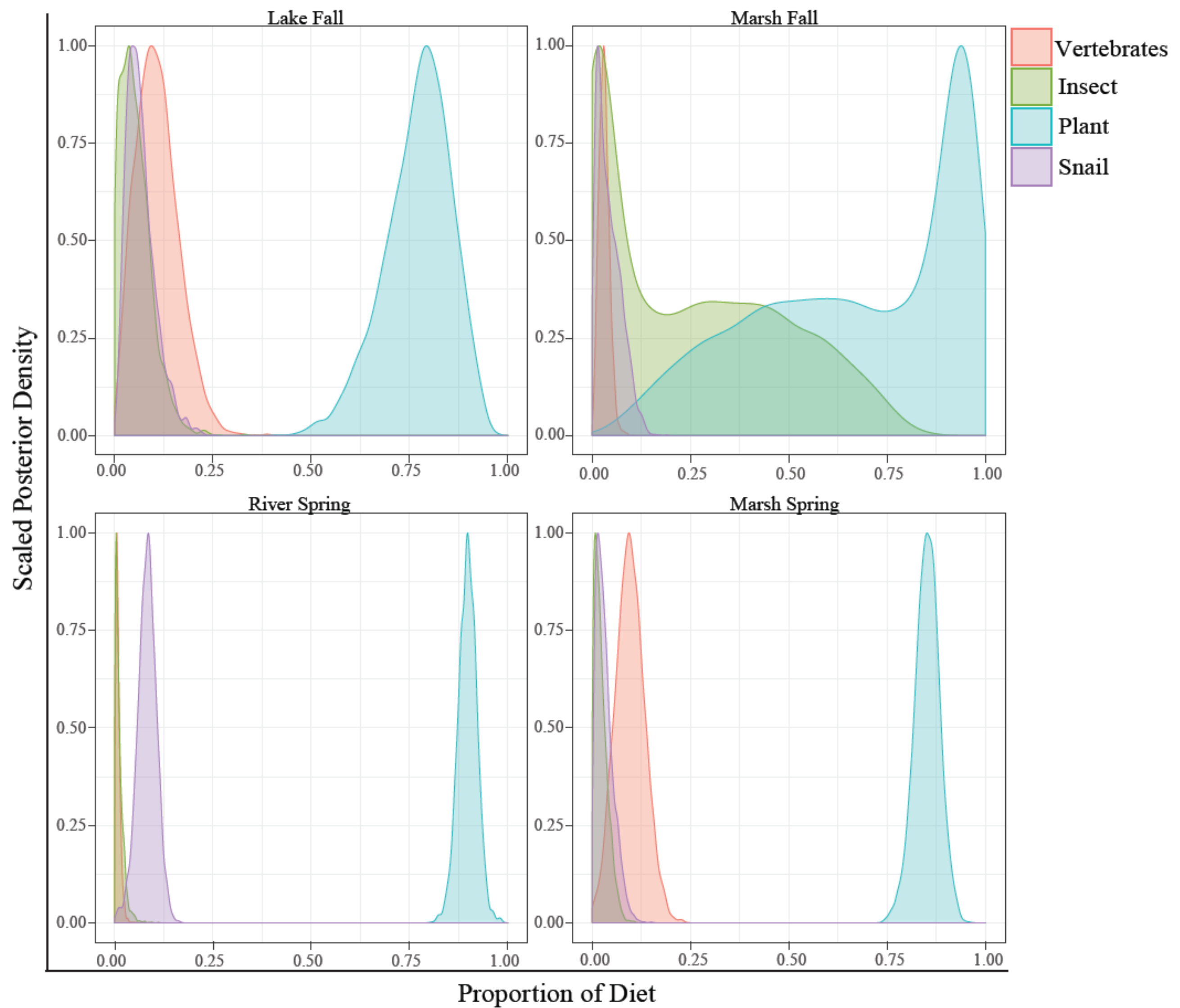


Figure 3.2. Posterior distributions of diet estimates for four groups of muskrats from southern Manitoba determined using stable isotope mixing models employing an uninformative prior, diet-tissue discrimination factor from Arneson and MacAvoy, 2005 (DTDF1) and concentration dependence parameter 2 (CD2).

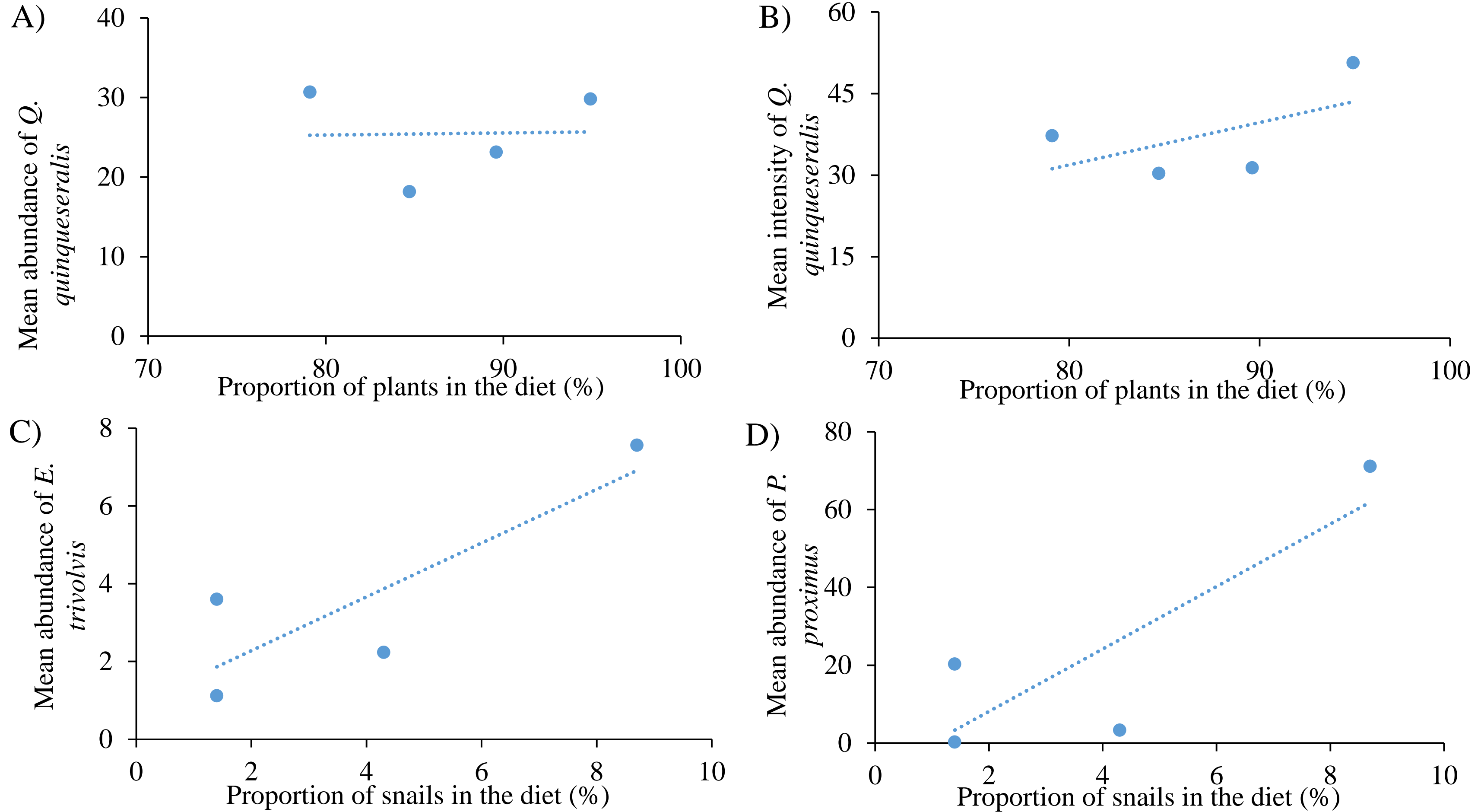
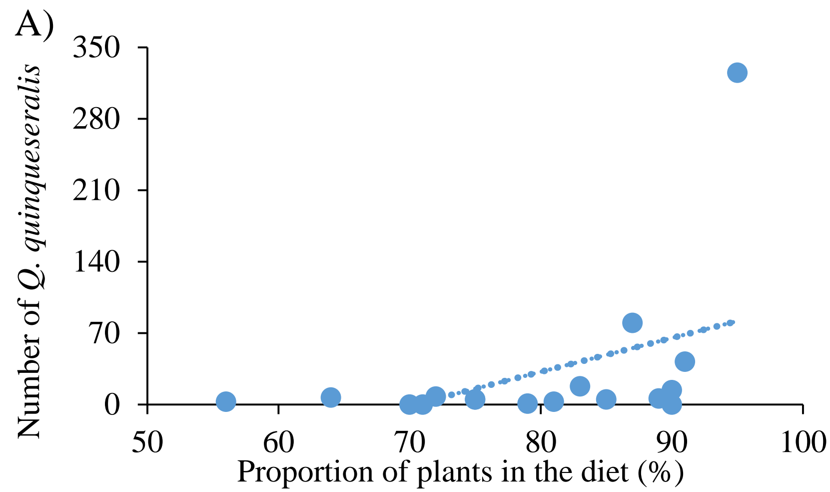
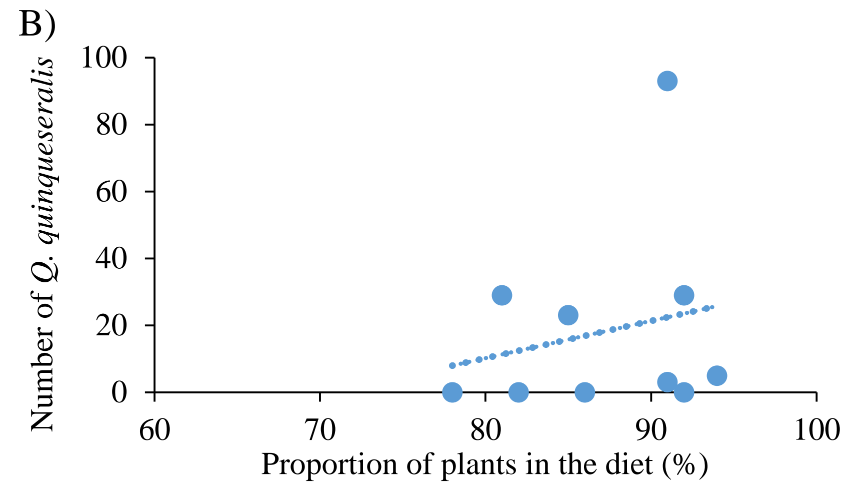
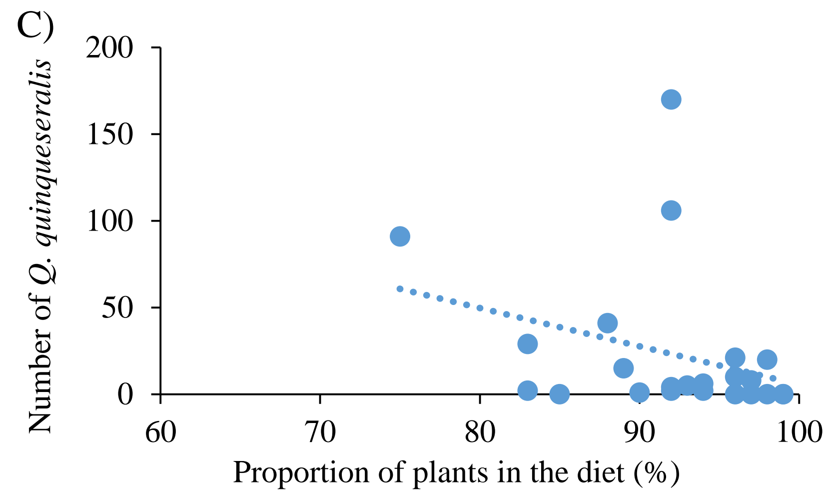


Figure 3.3. Linear regressions for proportion of food items in the diet and parasite abundance and intensity from four groups of muskrats. A–B) Proportion of plants and *Quinqueserialis quinqueserialis*, C) Proportion of snails and *Echinostoma trivolvis* parasites, D) Proportion of snails and *Plagiorchis proximus* parasites.







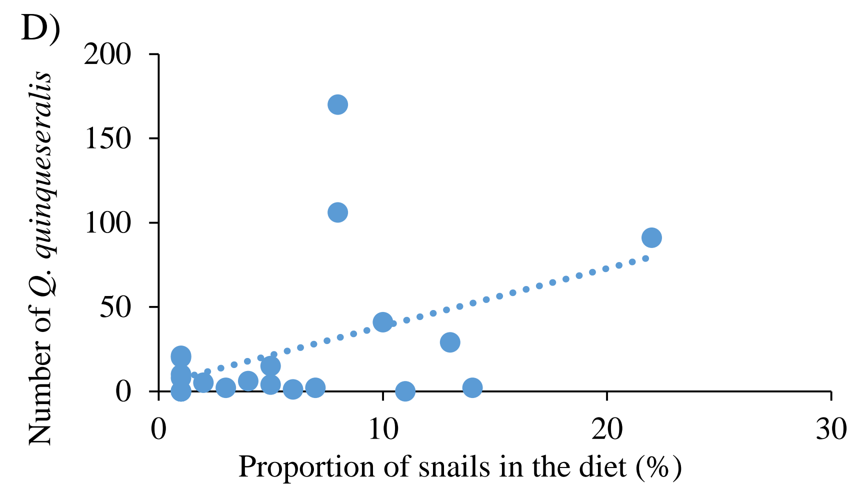


Figure 3.4. Linear regressions for proportion of food items in the diet and parasite intensity from individual muskrats from each of the three groups. A) Lake in Fall 2014; B) Marsh in Spring 2015; C–D) River in Spring 2015.

Thesis Conclusion

We tested a series of assumptions and parameters used in stable isotope analysis including DTDFs, turnover rates and allocation of nutrition sources to tissue synthesis to make stable isotope analysis a stronger tool in ecological studies. Chapter 1 expanded the number of taxa with known values of DTDF and turnover rates for carbon and nitrogen. By focusing on freshwater gastropods, we filled gaps in our knowledge about invertebrates as called for by several reviews (Caut *et al.*, 2009; del Rio *et al.*, 2009). Our study shows that $\Delta^{13}C$ is better predicted by values generated with species living in similar environments (i.e. freshwater, marine or terrestrial), whereas $\Delta^{15}N$ is best predicted from averaging values from several closely related species with similar diets. In general, freshwater snails had fast turnover rates, with half-life less than seven days for $\delta^{13}C$ and below 14 days for $\delta^{15}N$. This result suggests that snails reflect environmental isotopic change in short time periods and as primary consumers in food webs can provide the isotopic baseline for trophic position estimates. For DTDFs and turnover rates, we found that snail muscle and gonad tissue had different values, which suggested that invertebrates are similar to vertebrates in that tissue-specific values should be considered when estimating DTDF and turnover rates. By feeding snails two different diets, we found that diet isotopic values could cause DTDFs to be negative, which has been observed in other studies (Caut *et al.*, 2009). The most significant finding from Chapter 1 is that diet also affected growth performance, which in turn affected DTDFs and turnover rates. This result has significant implications for field studies of wildlife diet. In nature, the preferred diet may not always be available suggesting that DTDFs and turnover rates may change depending on the season and food availability.

To better understand factors that influence DTDF, Chapter 2 used isotopically distinct dietary sources to quantify the allocation of nutrition sources to tissue synthesis. We confirmed

that the proportion of carbohydrate and protein in the diet influenced DTDFs. Further, we found that this influence was because the contribution of these dietary nutrition sources to tissue synthesis was positively related to the concentration of the source in the diet. Feeding habits also affected the allocation of nutrition sources to tissue synthesis with generalist feeding species more likely to assimilate protein compared to herbivorous species. In addition, our study demonstrated that dietary carbohydrate can be an important source for tissue isotopic signal especially for primarily herbivores, though previous studies only suggested the role of protein (MacAvoy *et al.*, 2005; Arneson *et al.*, 2006). Novel equations allowed us to calculate ratios of incorporation rates between protein and carbohydrate in different diets, which is a step towards quantifying incorporation rates of dietary nutrition sources. By building on this approach, future studies should be able to better calculate concentration dependence in stable isotope analysis.

Using our knowledge gained from Chapters 2 and 3 on parameters and assumptions of stable isotope analysis, we integrated this approach with parasitism to better understand wildlife diet, parasite life cycles, and parasite transmission. Parasite infection patterns suggested potential food and prey items to sample for diet reconstruction that had not been emphasized in other studies such as freshwater snails, insects and tadpoles. Stable isotope analysis estimated the proportion of particular food items in muskrat diet at the group and individual levels. Linear regressions between the proportion of food items in the diet and parasite infection data showed some predicted relationships based off of life cycle information, but also suggested novel modes of transmission for some parasites. In one site, a plant-encysting parasite *Q. quinqueserialis* increased when the proportion of snails increased, suggesting that this parasite not only uses plants for transmission but also the surface of snail shells. These results demonstrated that stable

isotope analysis confirms and fills gaps in our knowledge of parasite life cycles and transmission.

Overall, our studies improve the reliability of wildlife diet reconstructions from stable isotope mixing models by generating a better understanding of the factors that influence DTDFs, turnover, and allocation of nutritional sources. Moreover, we demonstrated that stable isotope analysis can be integrated with parasitism to fill gaps in our knowledge of parasite life cycles and transmission. This information is critical for understanding the role of particular hosts in disease transmission and wildlife epidemiology.

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Appendix A: Calculating concentration dependence for food items in stable isotope mixing models for muskrats.

Macronutrient data of muskrat food items including cattail, snail, fish and frog were from the USDA nutrient database (www.nal.usda.gov/fnic/foodcomp/search) and shown in Table A.1. We focused on protein and carbohydrate because dietary lipids mainly synthesize lipids in consumers, and in our study, lipids were removed from muskrat and prey tissues.

The proportion of protein and carbohydrate in dry weight was calculated (Table A.2) based on the wet weight in Table A.1. Proportions of protein and carbohydrate dry weight were calculated by dividing the wet weight by the dry matter (1 - wet weight proportion of water). For the vertebrate (fish and frog) group, we calculated average values from three fish and one frog. These data were used to calculate the concentration of nitrogen and carbon in digestible matter (digest [N], digest [C], Table A.3). In each diet, we assumed that protein was 16% nitrogen and 52% carbon; carbohydrate was 0% nitrogen and 45% carbon (Robbins, 1993). Digestibility of protein was assumed to be 92% for plant and 100% for animal as recommended by Robbins (1993). Digest [C] was the sum of digestible protein [C] and carbohydrate [C]. There was no available macronutrient data for insects, so the average digestibility of [N] and [C] values from snail, vertebrates were used because the total % carbon and % nitrogen in these prey items was similar to insects based on our data. Specifically, the following equations were used:

Protein [N] = protein (% dry weight) $\times$ 0.16

Digest [N] = Protein [N] $\times$ 0.92 for plant; 1 for animal

Protein [C] = Protein (% dry weight) $\times$ 0.52

Digest Protein [C] = Protein [C] $\times$ 0.92 for plant; 1 for animal

Carbohydrate [C] = Carbohydrate (% dry weight) $\times$ 0.45

Digest [C] = Digest Protein [C] + Carbohydrate [C]

Insects digest [N] and digest [C] = average of snail and frog + fish

Digested [N] and [C] was used as the first concentration dependence parameter (CD1). For the second concentration dependence parameter (CD2), we tried to accounted for assimilation rates (Table A.4). We assumed digested protein was completely assimilated in muskrats, and based on Robbins (2002), assimilated C:N ratio of forbs and grasses is 4.5:1. Thus we calculated plant assimilated [C] by multiplying the assimilated [N] by 4.5. Then, we were able to calculate the proportion of carbohydrate [C] incorporated into muskrat tissues. For assimilated [C] in animal prey, we added the carbohydrate [C] with the incorporation proportion to the digested protein [C]. Average assimilated [C] values from snails, frogs and fish were used for insects as above.

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Table A.1. Weight of water, protein and carbohydrate in food items for muskrats from the USDA nutrient database (g/100g wet weight).

Food item	Water	Protein	Carbohydrate
Cattail	92.65%	1.18%	5.14%
Snail, raw	79.2%	16.1%	2%
Catfish, raw	80.36%	16.38%	0
Pike, raw	78.92%	19.26%	0
Mix bass, raw	75.66%	18.86%	0
Frog leg, raw	81.9%	16.4%	0

Table A.2. Proportion of protein and carbohydrate by dry weight in food items for muskrats from the USDA nutrient database (%).

Food item	Protein	Carbohydrate
Cattail	16.05%	69.93%
Snail, raw	77.40%	9.62%
Catfish, raw	83.40%	0
Pike, raw	91.37%	0
Mix bass, raw	77.49%	0
Frog leg, raw	90.61%	0
Mean vertebrates (frog + fish)	85.72%	0

Table A.3. Calculated values needed to determine digested [N] and [C] for concentration dependence parameter 1 (CD1).

	Protein [N]	**Digest [N]**	**Protein [C]**	**Digest protein [C]**	**Carbohydrate [C]**	**Digest [C]**
Plant	2.57	2.36	8.35	7.68	31.47	39.15
Snail	12.38	12.38	40.25	40.25	4.33	44.5
Vertebrates	13.72	13.72	44.57	44.57	0	44.57
Insect		13.05				44.54

Table A.4. Calculated values needed to determine assimilated [N] and [C] for concentration dependence parameter 2 (CD2).

	Assimilated [N]	Protein [C]	Digest protein [C]	Carbohydrate [C]	Assimilated [C]
Plant	2.36	8.35	7.68	31.47	10.62
Snail	12.38	40.25	40.25	4.33	40.65
Vertebrates	13.72	44.57	44.57	0	44.57
Insect	13.05				42.61

Appendix B: Results from MixSIAR models with different concentration dependence and diet-tissue discrimination factors.

In Chapter 3, we reported the results of muskrat diet estimates from MixSIAR models with CD2 and DTDF1. However, we also ran models with CD1, DTDF1 (Table B.1, Figure B.1); CD1, DTDF2 (Table B.2, Figure B.2–B.3) and CD2, DTDF2 (Table B.3, Figure B.4). According to all models, muskrats mainly consumed plants in all groups except for the Marsh Fall group using CD1 and DTDF 2. The results from this model showed that the mode for the proportion of insects in the diet was higher than plants. However, the credible intervals were overlapping suggesting no difference in the proportions of these food items. We were not surprised at this finding because the isotopic signatures of insects and plants were indistinguishable in this muskrat group, but they were still considered different prey items because we wanted to estimate their proportions because they could transmit different parasites.

Among the models, DTDF shifted the mode and the order of the proportion of food items in the diet. The mode for the proportion of prey items increased, while the mode for the plants decreased when using DTDF2 compared to DTDF1. The order of the proportion of different sources shifted for Lake Fall and Marsh Fall groups. In both groups, snails had a higher contribution to the diet than vertebrates with DTDF2.

In general, concentration dependence shifted the mode of the proportion of each food item in the diet. The mode for the proportion of prey items decreased, while the mode for the plants increased when CD2 was used compared to CD1. Despite some shifts in the mode depending upon whether CD1 or CD2 was used, the order of the proportions for different sources was the same for different CDs.

Table B.1. Dietary contributions (%) (mean, mode and 95% credible interval) for muskrat groups from the MixSIAR model with concentration dependence parameter 1 (CD1) and diet-tissue discrimination factor from Arneson and MacAvoy, 2005 (DTDF1). Hosts were collected from three different sites (Marsh, Lake, and River) in two seasons (Spring 2015, Fall 2014).

Muskrat	Vertebrates			Insect			Plant			Snail		
groups	Mean	Mode	95% CI	Mean	Mode	95% CI	Mean	Mode	95% CI	Mean	Mode	95% CI
Lake Fall	17.0	17.4	5.8–30.9	8.8	4.3	0.6–23.4	62.0	64.1	41.2–82.1	12.2	10.8	3.6–25.6
Marsh Fall	6.7	5.0	2.0–12.7	33.6	0.3	0.2–71.3	52.5	85.7	14.1–88.7	7.1	5.4	1.7–13.1
Marsh Spring	28.2	28.4	16.5–39.9	4.2	3.4	0.3–11.2	61.4	63.8	51.2–72.4	6.3	3.8	0.8–15.2
River Spring	1.3	0.7	0.2–3.7	1.7	0.6	0.1–6.3	71.8	70.8	61.8–84.9	25.1	27.6	12.0–35.2

Table B.2. Dietary contributions (%) (mean, mode and 95% credible interval) for muskrat groups from the MixSIAR model with concentration dependence parameter 1 (CD1) and diet-tissue discrimination factor from Kurle *et al.*, 2014 (DTDF2). Hosts were collected from three different sites (Marsh, Lake, and River) in two seasons (Spring 2015, Fall 2014).

Muskrat	Vertebrates			Insect			Plant			Snail		
groups	Mean	Mode	95% CI	Mean	Mode	95% CI	Mean	Mode	95% CI	Mean	Mode	95% CI
Lake Fall	12.3	14.6	4.1–22.6	14.0	4.7	1.3–38.1	46.1	39.8	20.1–73.8	27.6	22.6	7.7–55.1
Marsh Fall	5.6	3.4	0.9–12.6	38.0	44.5	1.0–65.6	38.0	32.2	6.2–80.9	18.3	21.0	6.1–28.3
Marsh Spring	38.8	39.8	23.1–52.5	3.2	0.9	0.3–9.2	52.4	52.3	40.8–65.4	5.6	3.9	0.9–14.2
River Spring	1.3	1.0	0.2–3.8	2.2	0.7	0.2–7.2	64.7	64.0	53.9–80.7	31.8	34.1	16.4–42.4

Table B.3. Dietary contributions (%) (mean, mode and 95% credible interval) for muskrat groups from the MixSIAR model with concentration dependence parameter 2 (CD2) and diet-tissue discrimination factor from Kurle *et al.*, 2014 (DTDF2). Hosts were collected from three different sites (Marsh, Lake, and River) in two seasons (Spring 2015, Fall 2014).

Muskrat	Vertebrates			Insect			Plant			Snail		
groups	Mean	Mode	95% CI	Mean	Mode	95% CI	Mean	Mode	95% CI	Mean	Mode	95% CI
Lake Fall	8.9	9.0	1.9–17.7	12.7	7.0	1.5–30.7	62.2	56.1	38.6–82.4	16.2	9.7	4.1–37.5
Marsh Fall	3.1	2.6	0.6–6.3	32.6	21.0	0.8–61.7	50.6	43.9	10.8–93.1	13.6	16.9	1.3–26.4
Marsh Spring	12.7	14.1	1.7–23.1	2.7	1.0	0.3–7.3	80.6	80.7	72.1–89.1	4.0	1.5	0.6–9.9
River Spring	0.7	0.4	0.1–2.0	1.4	0.5	0.1–4.6	86.9	87.4	81.1–92.6	10.9	11.8	5.3–16.4

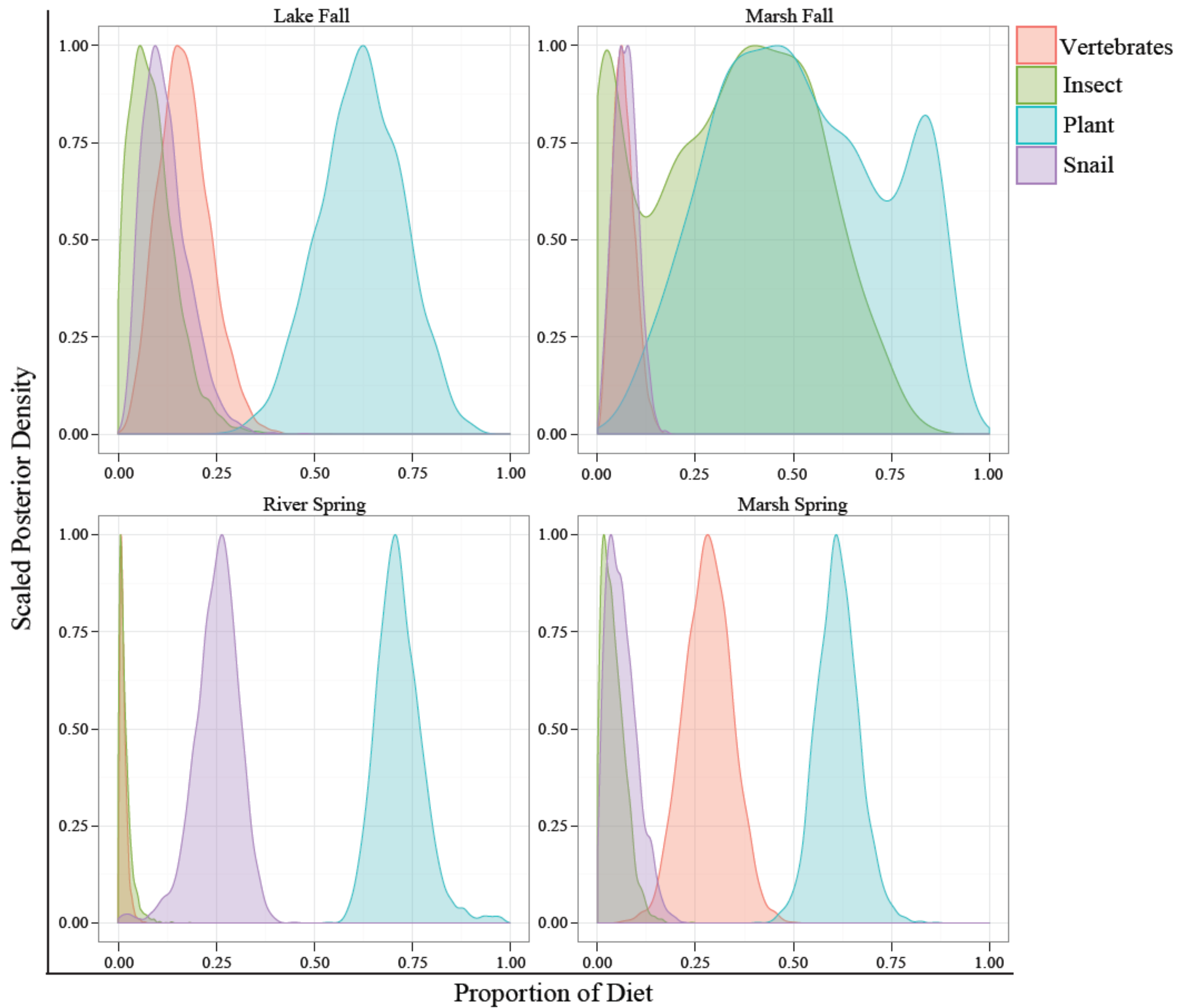

Figure B.1. Posterior distributions of diet estimates for four groups of muskrats from stable isotope mixing models employing an uninformative prior, concentration dependence parameter 1 (CD1) and diet-tissue discrimination factor from Arneson and MacAvoy, 2005 (DTDF1)..

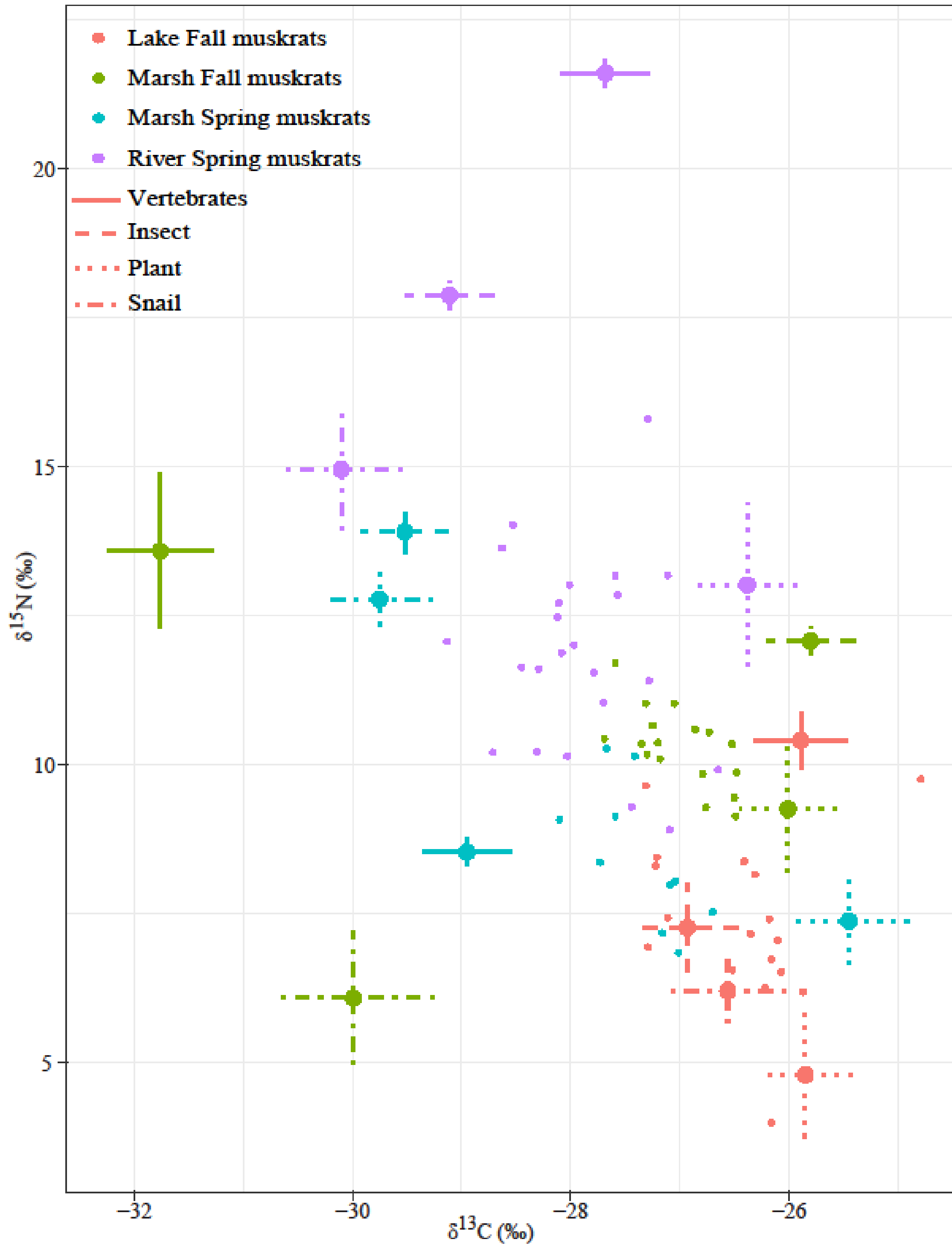

Figure B.2. Stable isotope plot of muskrats and prey items from four groups from southern Manitoba. Prey means were adjusted using diet-tissue discrimination factor from Kurle *et al.*, 2014 (DTDF2).

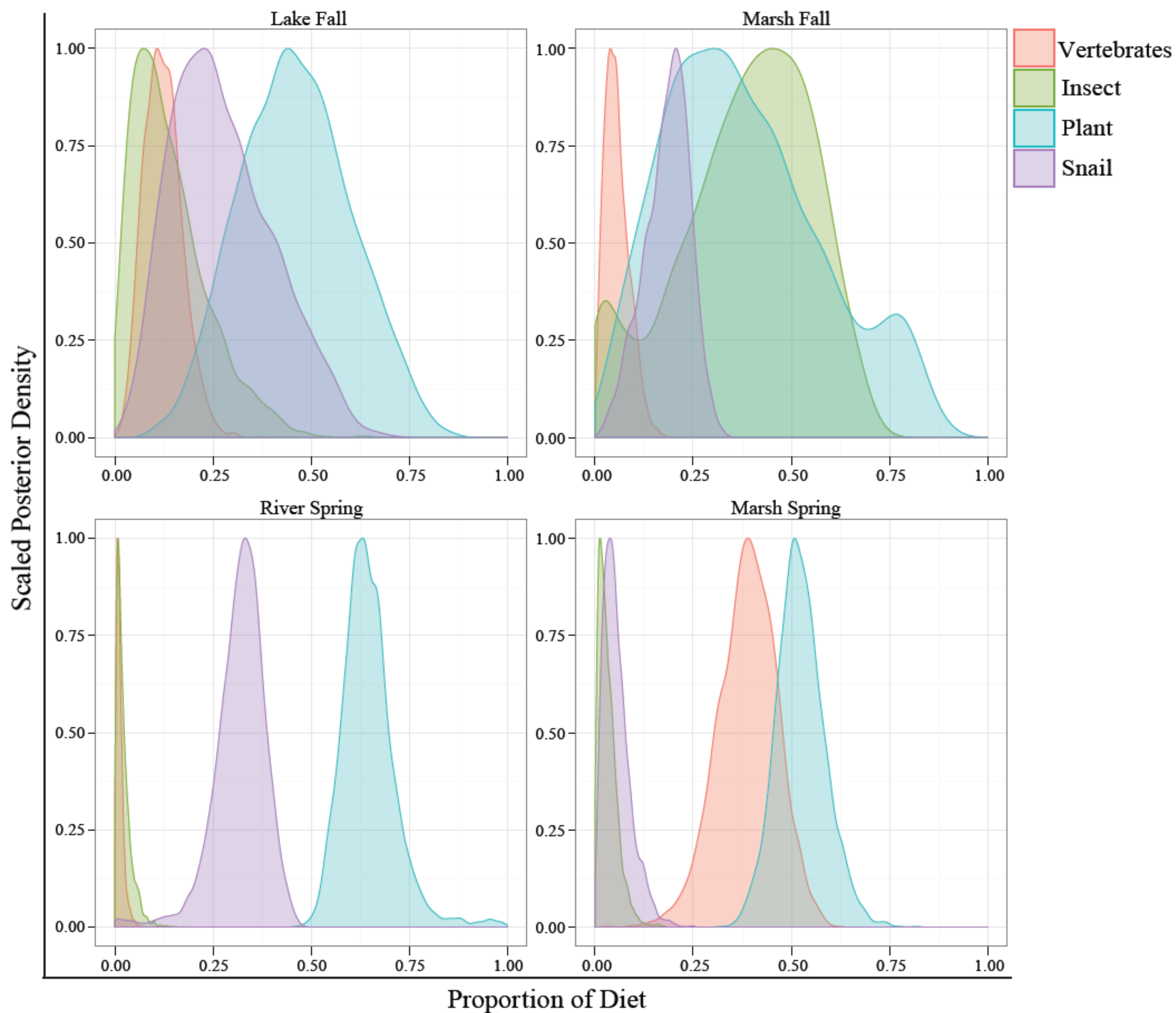

Figure B.3. Posterior distributions of diet estimates for four groups of muskrats from stable isotope mixing models employing an uninformative prior, concentration dependence parameter 1 (CD1) and diet-tissue discrimination factor from Kurle *et al.*, 2014 (DTDF2).

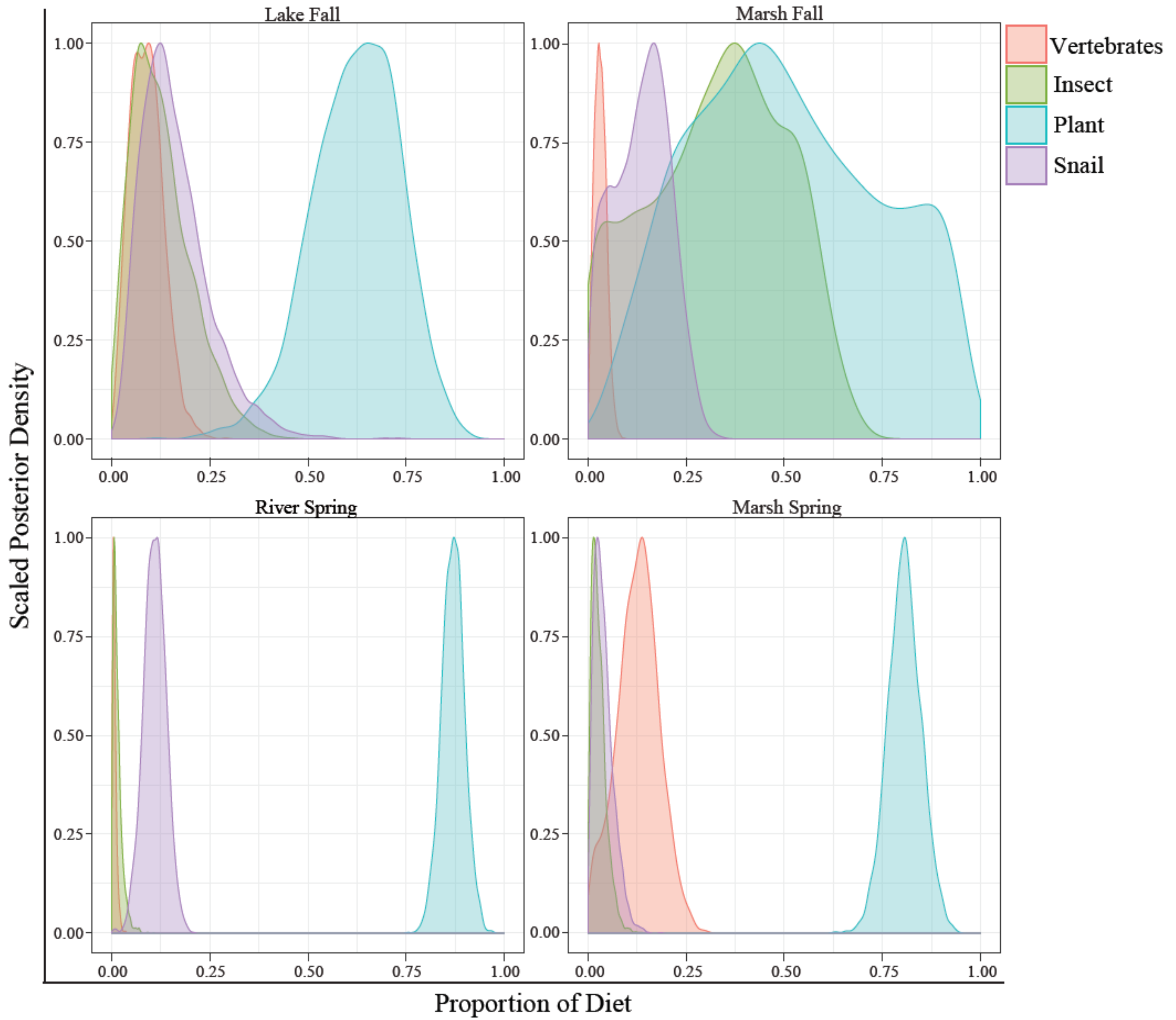

Figure B.4. Posterior distributions of diet estimates for four groups of muskrats from stable isotope mixing models employing an uninformative prior, concentration dependence parameter 2 (CD2) and diet-tissue discrimination factor from Kurle *et al.*, 2014 (DTDF2).

Table S.1. Stable isotope ratios of food item species for muskrats collected from three sites in southern Manitoba in Spring 2015 or Fall 2014.

Species	$\delta^{13}C$ (‰)	%C	$\delta^{15}N$	%N (‰)	n	Location	Season
Lithobates sylvaticus	-31.00	41.78	5.48	10.67	5	Marsh	Spring
Aeshna spp.	-31.55	46.72	11.15	12.53	3	Marsh	Spring
Sympetrum spp.	-31.59	42.72	10.54	11.09	1	Marsh	Spring
Lymnaea elodes	-31.52	37.10	9.32	9.54	3	Marsh	Spring
Helisoma trivolvis	-32.07	38.14	10.12	9.28	2	Marsh	Spring
Typha latifolia	-27.97	41.41	7.80	3.33	1	Marsh	Spring
Carex spp.	-26.83	41.45	3.72	2.51	1	Marsh	Spring
Phragmites spp.	-25.30	44.20	3.97	1.83	1	Marsh	Spring
Schoenoplectus tabernaemontani	-27.89	40.39	5.16	3.21	1	Marsh	Spring
Equisetum fluviatile	-26.23	39.10	4.96	1.99	1	Marsh	Spring
Aeshna spp.	-27.85	46.76	9.02	12.86	3	Marsh	Fall
Culaea inconstans	-34.09	42.95	9.24	13.50	2	Marsh	Fall
Ameiurus nebulosus	-33.53	42.78	11.81	13.41	1	Marsh	Fall

Helisoma trivolvis	-32.66	35.78	1.92	6.55	3	Marsh	Fall
Lymnaea stagnalis	-31.41	39.26	4.13	10.07	1	Marsh	Fall
Typha latifolia	-27.62	37.03	9.04	2.97	1	Marsh	Fall
Carex spp.	-26.63	32.53	6.49	4.25	1	Marsh	Fall
Equisetum fluviatile	-27.97	33.85	5.48	4.62	1	Marsh	Fall
Sympetrum spp.	-28.75	45.31	3.48	11.39	4	Lake	Fall
Aeshna spp.	-29.21	43.41	4.95	12.55	1	Lake	Fall
Pimephales notatus	-28.09	38.58	7.79	12.32	4	Lake	Fall
Culaea inconstans	-27.79	41.80	6.90	13.26	1	Lake	Fall
Helisoma trivolvis	-28.81	35.82	3.55	7.91	5	Lake	Fall
Lymnaea stagnalis	-27.93	40.95	2.15	10.87	2	Lake	Fall
Physa gyrina	-29.09	40.23	3.75	10.57	2	Lake	Fall
Typha latifolia	-27.99	41.22	0.57	1.87	1	Lake	Fall
Phragmites spp.	-26.74	44.58	4.17	0.44	1	Lake	Fall
Schoenoplectus tabernaemontani	-27.01	42.61	2.87	1.21	1	Lake	Fall
Pimephales promelas	-29.73	45.18	18.55	13.84	2	River	Spring

Corydalus spp.	-31.16	44.53	14.82	11.68	3	River	Spring
Helisoma trivolvis	-31.88	36.97	14.41	7.17	3	River	Spring
Lymnaea elodes	-33.28	41.07	9.68	11.11	3	River	Spring
Physa gyrina	-31.54	41.92	12.18	10.35	3	River	Spring
Promenetus spp.	-31.91	41.44	11.31	10.41	5	River	Spring
Carex spp.	-26.85	43.17	7.81	2.48	1	River	Spring
Equisetum fluviatile	-28.75	36.75	9.08	2.02	1	River	Spring
Schoenoplectus tabernaemontani	-28.03	39.40	13.48	3.23	1	River	Spring
Typha latifolia	-27.49	39.76	12.69	2.92	1	River	Spring