TURNOVER RATES AND TROPHIC DISCRIMINATION FACTORS OF $\delta^{34}S$ IN BROOK TROUT, *Salvelinus fontinalis*

by

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ABSTRACT

Stable isotope analysis (SIA) of sulphur, like carbon and nitrogen SIA, can be applied to understand food webs, animal movements, and nutrient dynamics. Sulphur SIA is of particular interest for movement studies of anadromous fishes because of the large variation between freshwater and marine sulphur ($\delta^{34}S$) stable isotope values. The application of this tool is dependent on our knowledge of the isotopic change between a consumer and its diet or the trophic discrimination factor ($\Delta\delta^{34}S$), and the turnover rate of the element in the organism's tissues. The objectives of this study were to determine the $\Delta\delta^{34}S$ and turnover rate of sulphur in liver, muscle, blood and caudal fin tissues, of brook trout (*Salvelinus fontinalis*), using laboratory and field experiments. At the start of the laboratory experiment, brook trout were fed a diet with a $\delta^{34}S$ value of 5.8±0.3‰, relative to their previous diet that had a $\delta^{34}S$ value of 15.9±0.2‰. For the field experiment, hatchery-reared brook trout were introduced to a New Brunswick stream, where their sulphur turnover rate and $\Delta\delta^{34}S$ were monitored *in situ*. These trout had an initial $\delta^{34}S$ value that was high relative to their natural diet in the stream. Subsequent sampling over several months permitted a comparison of natural and laboratory-derived turnover rates of sulphur in their tissues. The turnover rates from the laboratory results indicated the sulphur turnover was most rapid in liver and caudal fin and was slowest in blood and muscle. Similar turnover results were found in the field experiment. Laboratory measured $\Delta\delta^{34}S$ of those tissues that reached steady state averaged ~1‰ and the average $\Delta\delta^{34}S$ for all the tissues sampled from the Otter Brook resident brook trout in the field experiment was ~ 0 ‰. Results of this study will facilitate the use of sulphur as a third isotopic tracer in food web studies, and to successfully track fish movements.

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1.0 General Introduction

1.1 Stable Isotope Ecology

The earth is composed of chemical elements, and of these elements there are five that are most useful to stable isotope ecology. These elements are carbon (C), nitrogen (N), hydrogen (H), oxygen (O) and sulphur (S). What makes these elements useful for ecological application is that (1) these elements are found abundantly in the environment and in organisms, (2) these elements have more than one stable isotope, (3) the stable isotopes of these elements are found in variable ratios in both organisms and the environment, and (4) the stable isotopes of these elements can be measured using continuous flow isotope ratio mass spectrometry with accuracy and precision.

Stable isotopes are forms of an element with varying number of neutrons that do not decay over time. For example, the stable isotope versions of carbon are ^{12}C and ^{13}C, each with 12 and 13 neutrons, respectively. The heavy isotope (e.g., ^{13}C) is found in much lower abundance than the light isotope (e.g., ^{12}C) on Earth. The difference in mass between isotopes of the same element is due to the different number of neutrons. This results in differences in the kinetic reaction rate and the energy required for an isotope to take part in a chemical reaction, leading to a variation between the ratio of heavy and light isotopes from source to product as the isotopes move through ecosystems. The isotopic composition of these five elements changes in a predictable way as they cycle through the components of the biosphere (Peterson and Fry 1987).

Stable isotope ratios are measured using isotope ratio mass spectrometry (IRMS). The results of this method are expressed as the ratio of the heavy isotope to light isotope (e.g. $^{13}C/^{12}C$) in delta notation (e.g., $\delta^{13}C$) as the difference from an internationally set standard. Using isotope ratio mass spectrometry, ecologists can measure the stable isotope ratios of the components of the biosphere (i.e., air, water, sediment, plant tissues, animal tissues) and extract information on the chemical/biological processes and ecological relationships that govern it.

To be able to interpret the stable isotope results of a field study, ecologists need to know two biological parameters: (1) the rate that isotope ratios change in a consumer's tissues when a consumer shifts between diets with distinct stable isotope ratio values (i.e., turnover rate); (2) the difference between the stable isotope ratios of the consumer's tissues and its diet when the consumer reaches a steady state with its diet (i.e., trophic discrimination factor (TDF)). Tissue turnover rates record changes in diet and trophic discrimination factors are used as food source and trophic level indicators for consumers. There has been a continual request for a better understanding of what dictates turnover rates and TDF values since the establishment of the application of stable isotopes in ecology (e.g., Gannes *et al.* 1997, Grey 2006, Inger and Bearhop 2008; Wolf *et al.* 2009).

1.2 Isotopic Turnover Rates

The isotopic turnover rate of a given consumer's tissue refers to a measure of change, based on time or relative weight gain, in the isotope value of the consumer's tissue in response to a change in the isotope value of its diet. Growth (tissue addition) and metabolic turnover (tissue replacement) are the factors that drive stable isotope turnover rates (Fry and Arnold 1982; Hesslein *et al.* 1993). In terms of sulphur turnover rates, the addition and replacement of proteins in an organism's tissues reflect isotope turnover rates (Hesslein *et al.* 1993). All proteins in organisms' tissues are continuously replaced, but the rate of replacement varies between tissues and between proteins (Hawkins 1991). In other words, protein metabolic rates differ from one tissue to another. As protein metabolic rates vary between tissues, then the metabolic component of isotopic turnover rates will vary between tissues as well, giving rise to distinct turnover rates for each tissue. This has important implications in stable isotope ecology.

Ecologists should be mindful of the variation in isotopic turnover rates of tissues when designing their experiments and interpreting their results. Perga and Gerdeaux (2005) noted that whitefish (*Coregonus lavertus*) muscle tissue reflected diet during periods of growth, while liver tissue represented diet throughout the year. The difference in diet representation of these tissues was due to the greater metabolic contribution to isotopic turnover rates in liver than in muscle for which growth was the dominant cause for isotopic turnover. Metabolically active tissues, such as liver and blood plasma record short term changes in diet, while less metabolically active tissues such as muscle and collagen record long term dietary information (Tieszen *et al.* 1983; Hobson and Clark 1992). Choosing the appropriate tissue that will record the information required to address the research objectives is dependent on our knowledge of the rates of isotopic incorporation for each tissue. Besides contributing to sound experimental design, knowing the isotopic turnover rates of several tissues for the same organism can determine finer-scale events such as when a change in diet has occurred. The "isotopic clock" concept is based on the idea that sampling different tissues with different isotopic turnover rates makes it possible to determine when a change in diet has occurred (Philips and Eldridge 2006; Heady and Moore 2013).

1.3 Trophic Discrimination Factors

The trophic discrimination factor is the difference between the stable isotope ratio value of a consumer's tissue and its diet. Trophic discrimination factors are an absolute requirement to the proper application of SIA in ecology. If this parameter is not well quantified, our ability to determine food sources and trophic levels via SIA, with certainty, is not possible. Trophic discrimination factor values vary between elements, organisms, tissues of organisms and are affected by diet – same organisms with different quality and/or quantity of a diet can result in different tropic discrimination factor values (reviewed in: McCuthan *et al.* 2003; Martinez del Rio *et al.* 2009). McCutchan *et al.* (2003) fed buckeye butterflies, *Junonea coenia*, three different diets at both the pupae and adult life stages. The TDF values for the pupae and adult buckeye butterflies on the three different diets varied (carbon: -0.7 to -2.7‰, nitrogen: 0.9 to 3.6‰, sulphur: -1.5 to 7.3‰).

1.4 C, N, H, O and S

Carbon is often used as a food source indicator due to the minimal fractionation that occurs between prey and consumers, $\Delta\delta^{13}C$ values of 0-1‰ (DeNiro and Epstein 1978; Peterson and Fry 1987; Post 2002). The distinct $\delta^{13}C$ values between C3 and C4/CAM plants also allows for the distinction between the primary sources of carbon to an organism's diet (Marshall *et al.* 2007). Nitrogen is typically used as a trophic level indicator due to the predictable enrichment (i.e., increase of the $\delta^{15}N$ value) with each increase in trophic position (Fry 2006). Often, the $\Delta\delta^{15}N$ for nitrogen is cited to be 3.4‰ (Post 2002), but it can vary due to factors such as protein quality, food quantity and methods of nitrogen excretion (reviewed by: Koch 2007; Hobson 2008). Due to the predictable nature of δ^2H (or δD) and δ^{18}O on continental landscapes, hydrogen and oxygen are used for terrestrial animal migration studies (e.g., Bowen *et al.* 2005). Sulphur is often used to distinguish between terrestrial and marine dietary sources in estuaries (Wassenaar 2008), but also has potential as a food source indicator within estuaries and lakes (Peterson *et al.* 1985; Croisetière *et al.* 2009), as well as application for fish movements (Fry and Chumchal 2011). There are four stable isotopes for sulphur that are at different relative abundances: ^{32}S (95%), ^{33}S (0.75%), ^{34}S (4.21%), ^{36}S (0.014) (Sulzman 2007). For sulphur stable isotope analysis, ^{32}S and ^{34}S are the light and heavy isotopes, respectively, used most commonly for determination of the stable isotope ratio of δ^{34}S.

1.5 Sulphur in the Biosphere

As mentioned above, δ^{34}S values for marine and terrestrial systems are distinct. The δ^{34}S for ocean sulphate is 21‰ (Rees *et al.* 1978). Terrestrial and freshwater δ^{34}S values vary between -22 and 20‰ (Peterson and Fry 1987), but are typically lower than that of ocean sulphate (Wassenaar 2008). The distinct δ^{34}S values between marine and continental systems is evident in primary producers: continental vegetation typically have an average δ^{34}S value between 2 and 6‰, whereas marine plankton and seaweeds typically have a δ^{34}S value between 17 and 21‰ (Peterson and Fry 1987). Terrestrial δ^{34}S values are considered as a bedrock patchwork caused by the uplifts of marine sediment and evaporates which vary in their δ^{34}S value. The δ^{34}S value variation of these uplifts is due to the variation in the degree of sulphur fractionation due to sulphate reduction by sulphate reducing bacteria on a geological time scale (reviewed by Peterson and Fry 1987).

The $\delta^{34}S$ value of primary producers, such as plants and algae, vary depending on their primary source of inorganic sulphur (Peterson *et al.* 1985; Krouse *et al*. 1991) and the level of sulphur reduction by bacteria in the primary producer's environment (Peterson 1999; Canfield 2001). For example, Peterson *et al.* (1985) noted that upland plants ($\delta^{34}S$ =4.7±0.9‰) that uptake sulphate from aerobic soils were more enriched than the salt marsh grasses ($\delta^{34}S$ =-2.4±4.4‰) that obtained their sulphate from anoxic sediment. The latter sediment typically has higher levels of bacterial sulphate reduction than aerobic soils. Planktonic algae ($\delta^{34}S$ =18.8±0.6‰) that take up sulphate from seawater had a similar $\delta^{34}S$ value to ocean sulphate (Peterson *et al.* 1985).

The $\delta^{34}S$ value of animal tissues is assumed to resemble that of its diet. In animal tissues, sulphur is present in proteins, specifically, in the amino acids methionine and cysteine (Krouse *et al.* 1991; Koch 2007; Hobson 2008). Methionine is an essential amino acid and therefore can only be acquired via diet, whereas cysteine is considered conditionally-essential since it can either be acquired via diet or biosynthesized from methionine (Krouse *et al.* 1991; Koch 2007). Since methionine and cysteine are essential and/or derived from essential amino acids, there is little opportunity for these amino acids to be isotopically modified during assimilation. The $\Delta\delta^{34}S$ values associated with sulphur are considered to be negligible despite the lack of reliable evidence (Peterson and Howarth 1987; Hesslein *et al.* 1991).

1.6 Ecological Applications of Sulphur SIA

Due to the natural gradient of $\delta^{34}S$ values from freshwater through estuaries to the sea (Fry and Chumchal 2011), and the utility of sulphur to distinguish between primary producers (Fry *et al.* 1982; Peterson *et al.* 1985; Connolly *et al.* 2004;

Croisetière *et al.* 2009), sulphur SIA has great potential in aquatic food web studies and in fish movement studies. For example, sulphur SIA has shown to be useful in tracing marine derived nutrients into tidal freshwater ecosystems (MacAvoy *et al.* 1998). In terms of isotopically distinguishing between primary producers in estuaries and marine based food webs, combining sulphur and carbon is more useful than combining nitrogen and carbon (Peterson *et al.* 1985; Connolly *et al.* 2004). In estuaries, sulphur SIA has been successfully applied to identify resident and transient fishes (Fry and Chumchal 2011).

Stable sulphur isotope analysis has been used in numerous ecological studies, such as identifying pollution sources in lichen (Wadleigh 2003), identifying the diets of grizzly bears (*Ursus arctos horribilis*) (Felicetti *et al.* 2003), and tracing marine derived nutrients in freshwater tidal ecosystems (MacAvoy *et al.* 2001). Sulphur has not yet been used to its full potential in food web and migration studies. The considerable lack of knowledge about turnover rates in animal tissues and TDFs makes sulphur isotope analysis an important area of study. My thesis aimed to address this shortcoming by performing a laboratory and field diet shift study on brook trout, *Salvelinus fontinalis,* to determine (1) turnover rates and (2) trophic discrimination factors in several fish tissues.

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2.0 Turnover rates and trophic discrimination factors of $\delta^{34}S$ values in brook trout, *Salvelinus fontinalis*, determined by both field and laboratory experiments

2.1 Abstract

Sulphur stable isotope ratios in marine and freshwater systems are typically distinct from each other and can therefore be used as a tool in migratory fish studies. To exploit the distinct sulphur isotope ratios of marine and freshwater systems in migratory fish studies, sulphur turnover rates in fish tissues and the difference of sulphur isotope ratios between a fish's tissue and its diet (trophic discrimination factor, $\Delta\delta^{34}S$) are required information. Diet shift experiments in the laboratory and the field were performed to determine the turnover rate of sulphur in brook trout (*Salvelinus fontinalis*) liver, muscle, blood and caudal fin tissues. Results from the laboratory experiments indicated that sulphur turnover was most rapid in liver (0.037±0.008 ‰ • day^{-1}) and caudal fin (0.040±0.002 ‰ • day^{-1}), and was slowest in blood (0.015±0.004 ‰ • day^{-1}) and muscle (0.011±0.007 ‰ • day^{-1}). Similar results were found in the field experiments where hatchery-reared brook trout were introduced to a stream and periodically sampled over five months. The $\Delta\delta^{34}S$ values of the tissues in the laboratory that reached steady state averaged ~1‰ and the average $\Delta\delta^{34}S$ for the tissues sampled from the resident brook trout in Otter Brook was ~ 0‰. The tissue-specific sulphur turnover rates, the low $\Delta\delta^{34}S$ values, and the substantial difference between marine and freshwaters $\delta^{34}S$ values imply that sulphur will be a useful tracer for migratory fish movements between freshwater and the marine environment.

2.2 Introduction

Stable isotope analysis (SIA) is a powerful analytical method in ecology. The stable isotope values of an element are useful to ecologists when (1) there exists a natural variation in the stable isotope values in the environment and (2) biochemical processes in animals create variation in food webs. Five elements exhibit natural variation in their stable isotope values in the environment and in food webs and they are hydrogen, carbon, nitrogen, oxygen and sulphur (Fry 2006). Carbon, nitrogen and hydrogen are the most commonly used elements in stable isotope ecology (Barnes and Jennings 2007; Crawford *et al*. 2008; Layman *et al.* 2011). The carbon isotope ratios ($\delta^{13}C$) of a consumer and its diet are similar (Deniro and Epstein 1978; Peterson and Fry 1987), thus carbon stable isotope analysis is often used as a food source indicator. Nitrogen isotope values are often used as an indicator for an animal's trophic position within a food web because nitrogen isotope values ($\delta^{15}N$) of a consumer can be enriched on average by 3-5‰ relative to the average diet value (DeNiro and Epstein 1981; Peterson and Fry 1987). The predictable relationship between a consumer's carbon and nitrogen isotope composition and that of its diet, as mentioned above, does not hold true in all situations. McCutchan *et al.* (2003) compiled the isotope values of consumers relative to their diet for carbon and nitrogen from published studies. They found that the carbon isotope value difference between a consumer and its diet can range between -2.7‰ to +3.4‰ and nitrogen can range from -0.8‰ to 5.9‰. Due to the predictable geographical variation of δ^2H (or δD) values on the continental landscape, hydrogen is often used for animal migration studies (Bowen *et al.* 2005). Carbon, nitrogen and hydrogen are used most often because the analytical methods are the most established, whereas the methods required for oxygen and sulphur analysis are not yet routine in most stable isotope analytical laboratories.

Natural variation in sulphur stable isotope ratios ($\delta^{34}S$) occurs in aquatic systems at several levels (Croisetière *et al.* 2009; Fry and Chumchal 2011). Ocean sulphate is considered constant at a $\delta^{34}S$ value of 21‰ (Rees *et al*. 1978). Freshwater systems are more variable in their sulphur isotopic values ranging between -22 to 20‰ and trend towards being depleted in ^{34}S relative to the ocean sulphate (Fry 2006; Wassenaar 2008). The resulting natural gradient of water sulphate $\delta^{34}S$ values can be useful to interpret fish movements (Fry and Chumchal 2011), especially for diadromous fishes. The $\delta^{34}S$ values of primary producers also vary in aquatic systems (Fry *et al.* 1982; Peterson *et al.* 1986; Connolly *et al.* 2004; Croisetière *et al.* 2009) due to the differences between the water column and sediment sulphur pools available for uptake (Peterson 1999; Canfield 2001).

The successful application of sulphur stable isotope analysis to fish movement studies requires knowledge of the turnover rate of $\delta^{34}S$ in the tissues of fish as well as the trophic discrimination factors ($\Delta\delta^{34}S$). Stable isotope turnover in tissues relates to protein metabolism and protein addition from growth (Hesslein *et al*. 1993). Protein metabolism varies between tissues (Tieszen *et* al. 1983; Hawkins 1991) and therefore, can provide information over different time periods regarding changes in dietary sources. The $\Delta\delta^{34}S$ is the difference in the stable isotope value of an animal's tissues and its average diet. The $\Delta\delta^{34}S$ for consumers is assumed to be negligible (Hobson 2008). Barnes and Jennings (2007) found that the average $\Delta\delta^{34}S$ of European sea bass, *Dicentrarchus labrax*, relative to its diet, was -0.5±0.1‰. However, other controlled laboratory diet shift studies have noted $\Delta\delta^{34}S$ values of 1.4‰ in brook trout, *Salvelinus fontinalis* (Peterson *et al.* 1985) and 1.5‰ in broad whitefish, *Coregonus nasus* (Hesslein *et al*. 1993). Suggesting a need for more diet shift studies.

Turnover rates and $\Delta\delta^{34}S$ values are most commonly determined through laboratory controlled diet shifts. Laboratory experiments are useful because (1) the isotopic composition, quantity, and quality of the diet can be controlled, (2) the start and duration of the diet shift can be controlled, and (3) often weight and length can be measured throughout the experiment, which are necessary data to understand how growth and metabolism contribute to isotopic turnover. Despite their usefulness and necessity, controlled laboratory diet shifts have limitations. Consumption rate, activity and dynamic environmental conditions are factors that can affect metabolism and growth in fish and these factors are typically held constant in controlled laboratory diet shifts. In the wild however, these factors are variable and can affect the turnover rates. Occasionally, researchers have measured tissue turnover in the wild, such as during ontogenetic diet shifts (Graham *et al*. 2007) or by tracing isotopically distinct food sources during the migration/movement of the consumer or prey species (MacAvoy *et al*. 2001). In the absence of such opportunities, it is sometimes possible to monitor a natural diet shift by transplanting animals to a new, isotopically distinct environment. In the present study, we stocked hatchery-reared brook trout into a small stream (with natural barriers to minimize movement) and periodically sampled fish to measure isotopic tissue turnover. Because the trout were reared on a pellet diet (marine origin), it was possible to trace their diet shift to freshwater food. We then compared the results from both the laboratory and field diet shift experiments.

Only four published studies have investigated sulphur turnover rates and/or $\Delta\delta^{34}S$ values in fishes (Peterson *et al.* 1985; Hesslein *et al.* 1993; MacAvoy *et al.* 2001; Barnes and Jennings 2007). By contrast, there are ~40 carbon, and ~50 nitrogen, controlled diet studies conducted on fish to determine TDF values (Caut *et al*. 2009). The objectives of my thesis were (1) to determine the turnover rates of, and $\Delta\delta^{34}S$ values associated with, liver, muscle, blood and caudal fin tissue of age 2+ brook trout via a controlled laboratory diet shift and (2) to compare the turnover rates and $\Delta\delta^{34}S$ values determined in the laboratory to a diet shift in a natural setting for similar aged brook trout. Comparisons between laboratory controlled diet shifts and diet shifts in a natural setting, when possible, are necessary to better understand the outcomes and applications of the results obtained from the two settings. The knowledge on how laboratory results can differ from, and translate into, field results is crucial to the proper interpretation of sulphur stable isotope data in fish movement/migratory studies.

2.3 Methods

2.3.1 Laboratory Experiment

The species chosen for this study, brook trout (*Salveninus fontinalis*) is a mobile opportunistic forager that is distributed from headwater tributaries to the river's estuary (Curry *et al.* 2002). This behaviour makes brook trout an ideal species for studies of fish movement because it can be feeding in habitats that vary in their $\delta^{34}S$ values. Hatchery reared age 2+ brook trout (mean±SD: 19.6±1.7cm fork length, n=443) from the Miramichi Salmon Conservation Centre were transferred to the aquatic facility at the

University of New Brunswick (UNB) where experiments were conducted according to the guidelines of the Canadian Council on Animal Care (CCAC/CCPA) and UNB (Approval # 11023, 12017, 13016). The laboratory experiment was carried out in four identical 350L cylindrical tanks each containing approximately 100 brook trout (26g/L). Room and water temperatures were regulated and the photoperiod was set at 12:12 hours of light:dark. Minimum water temperatures were maintained at 13°C with water heaters, but it was not possible to cool the water if temperature exceeded 13°C. The average water temperature throughout the laboratory experiment was 14.2±1.7°C (mean±SD, n=8071; min: 12.3°C, max: 18.5°C). All tanks were connected to a flow through system of dechlorinated freshwater. To obtain a diet with a depleted $\delta^{34}S$ value relative to the initial diet of the brook trout, a variation on the salmonid pelletized diet developed by Lall and Castell (unpublished) of Fisheries and Oceans Canada was produced. The original pelletized diet (produced with herring fishmeal) was replaced with that of a freshwater species, tilapia (*Oreochromis niloticus*) (Table 1). The brook trout were fed the customized pellet diet until satiation twice a day during weekdays and once a day on weekends and statutory holidays.

Prior to the diet shift, fish were measured for fork length (cm), weighed (g) and tagged with visual implant elastomer (VIE) tags (Frederick 1997), using a unique colour code. Prior to the first feeding at the start of the diet shift, five fish from each tank were lethally sampled to obtain their initial sulphur isotope composition (Table 2). At each subsequent sampling period, five fish from each tank were lethally sampled. Sampled individuals were identified, weighed and fork length measured to determine individual growth rate over the sampling period. During each sampling event, blood, liver, caudal fin clip, and muscle tissues were collected and oven-dried at 60°C for 72h. Blood was sampled from the ventral vein along the spinal column by inserted a 25½ gauge needle of a syringe just below the lateral line and in line with the anal thin. Caudal fin clips were collected from the distal section of the fin, and the muscle sample was taken from the epaxial musculature anterior to the dorsal fin. Samples of the diet (i.e., pellets) were also collected at two week intervals throughout the diet shift experiment and oven dried at 60°C for 72h. All ingredients contributing to the pelletized diet were collected and oven dried at 60°C for 72h. Both tissue samples and diet samples were kept in a freezer prior to stable isotope analysis.

2.3.2 Field Experiment

Hatchery reared age 2+ brook trout from the Miramichi Salmon Conservation Centre were collected to stock in a 500m section of Otter Brook, a second order tributary to the Little Southwest Miramichi (46°52'38.8"N, 66°02'09.9"W). Initial stocking density was 0.6 brook trout per m^2. Fish were marked with a VIE tag in the anal fin to differentiate hatchery brook trout from wild resident brook trout. Three size ranges were tagged: blue VIE tagged fish ranged from 7.5 to 10.9cm in fork length (mean±SD 9.7-±0.9cm, n=144); yellow VIE tagged fish, 11.0 to 14.9cm in fork length (13.3±1.1cm, n=959); and, red VIE tagged fish, 15.0 to 22.2cm in fork length (16.5±1.3cm, n=818). Three fish from each size-class were killed before stocking to obtain the initial $\delta^{34}S$ values (Table 3).

Before stocking in the lower 500m reach of the stream, some wild brook trout were removed to (1) increase the likelihood of successful establishment of the hatchery brook trout in the stocked area and (2) increase the likelihood of subsequent capture of the hatchery fish. These wild brook trout were transferred upstream of a beaver dam in Otter Brook. The hatchery-reared brook trout were stocked in Otter Brook on 04 June 2012. There were six sampling events from 12 June 2012 to 27 October 2012 on days 8, 16, 29, 47, 88, and 145 of the field experiment. An additional sampling was carried out on day 359 (29 May 2013) to assess growth and condition of trout a year after stocking. During each sampling event, a maximum of ten stocked brook trout and three wild brook trout were euthanized with 40% clove oil: ethanol applied at a dosage of 80 parts per million (clove oil and ethanol:water), followed by a strike to the head. Electrofishing was used to capture wild and hatchery brook trout. Weight and fork length measurements were collected for each fish. Liver, caudal fin tissue, and muscle tissue were collected and oven-dried at 60°C for 72h. Stream benthic macroinvertebrates taxa were collected during each sampling event for analysis as possible dietary items. Four benthic macroinvertebrate species were collected at each sampling event to represent three functional feeding groups: predators (Corydalidae, Rhyacophilidae), scrapers (Heptageniidae), and filter-feeders (Simuliidae).

2.3.3 Sulphur Stable Isotope Analysis

Oven-dried (60°C) blood, liver, and muscle tissue were ground to a homogenous powder, whereas caudal fin samples were analyzed whole. Samples of the pelletized diet were oven-dried at 60°C and ground to a homogenous powder. All samples were weighed out in tin capsules at 5.0 – 5.3mg. Approximately 5mg of vanadium pentoxide was added to samples and standards as a catalyst to promote combustion.

Sulphur stable isotope analyses was conducted at the Stable Isotope in Nature laboratory at the University of New Brunswick/Canadian Rivers Institute on a Costech 4010 (Milan, Italy) elemental analyser interfaced through a Thermo Finnigan (Bremen, Germany) Conflo II to a Thermo Finnigan (Bremen, Germany) Delta Plus continuous flow isotope ratio mass spectrometer. A heated gas regulator, secondary to the tank regulator, was placed between the SO_2 reference gas tank and the Conflo II open split to minimize the formation of sulphuric acid by removing water from the SO_2 gas flowing from the reference tank to the Conflo II. The results are reported in standard per mil (‰) δ notation vs. the primary ^{34}S isotope standard V-CDT (troilite of the Canyon Diablo meteorite). IAEA-S-1 (silver sulphide, $\delta^{34}S_{V\text{-}CDT}$= -0.3±0.2‰ (mean±SD)) and IAEA-S-2 (silver sulphide, $\delta^{34}S_{V\text{-}CDT}$= 22.7±0.7‰) standards were used to calibrate two internal laboratory standards (Squid Sulphur Standard $\delta^{34}S$= 19.6±0.2‰; Bass Check Standard $\delta^{34}S$= 3.8±0.2‰) to international standards. Sample correction was done using these two organic standards. Repeated measurements of these internal laboratory standards and a third internal laboratory check standard (Pollock Sulphur Standard $\delta^{34}S$= 17.9±0.3‰) indicated that the instrument precision (SD) was ±0.3‰.

2.3.4 Data Modeling

Three models were used for data analysis, (1) a growth-based model (Fry and Arnold 1982); (2) a time-based model (Hesslein *et al.* 1993); and 3) a multi-compartment reaction progress variable model (Cerling *et al.* 2007; Martinez del Rio and Anderson-Sprecher 2008). The growth-based model accounts for varying growth rates due to different environmental conditions but is limited by the necessity of growth data for its application. Growth data for fish in field experiments are not always available (i.e., due to logistic, time and resource constraints for sampling a population of fish through time) thereby making a growth-based model of limited value for field based studies. The time-based model is more applicable to field studies, because growth data are not required. Time-based turnover rates are considered appropriate if fish sampled in the field are experiencing similar environmental conditions and growth rates as the laboratory fish from which the turnover rates were estimated. The multi-compartment model is useful because it can determine the number of possible *isotopic pools* and their specific turnover rates (Cerling *et al.* 2007; Carleton et al. 2008). Isotopic pools can be described as different components of the bulk tissue being analysed. Different components of a sample of bulk tissue (i.e., plasma and red blood cells in whole blood) can have varying turnover rates (Hawkins 1991). Carleton *et al.* (2008) observed that the multi-compartment models typically estimated slower turnover rates and smaller $\Delta\delta^{34}S$ values than the one-compartment models. Determining how many isotope pools are contributing to isotope turnover allows for the use of a model that takes the number of isotope pools into account. This results in a model that best describes the data.

Fry and Arnold (1982) applied a turnover rate model based on relative growth, herein referred to as the *growth-based model*:

$$\delta = \delta_f + (\delta_i - \delta_f)(w_t/w_i)^{(c+1)}$$

where $\delta_f = \delta^{34}S$ value at steady state with the diet, $\delta_i =$ initial $\delta^{34}S$ value, $w_i =$ initial fish weight (g), $w_t =$ fish weight on the day it was lethally sampled, $\delta =$ the $\delta^{34}S$ value at weight w_t, and c is the rate of $\delta^{34}S$ turnover relative to w_t/w_i. When $c = -1$, $\delta^{34}S$ changes are due to tissue addition diluting the initial $\delta^{34}S$ and when $c < -1$ $\delta^{34}S$ changes are due to dilution as well as metabolic turnover.

Hesslein *et al*. (1993) applied a model where the turnover rate was measured relative to time from the start of the diet shift, herein referred to as the *one-compartment time-based model*:

$$\delta = \delta_f + (\delta_i - \delta_f)e^{-\lambda t}$$

where t = days after the diet shift and λ = the turnover rate of $\delta^{34}S$. If growth is measured, the contribution of turnover due to metabolism (m) and due to growth (k) can be determined, $\lambda = m + k$ (see Hesslein *et al.* 1993).

Cerling *et al.* (2007) used a reaction progress variable (RPV) method to determine the number of isotopic pools contributing to turnover. This method is similar to that of Hesslein *et al*. (1993) as it follows first order rate kinetics, but differs by normalizing and linearizing the isotope dataset. Using the Cerling *et al.* (2007) method we can determine if there are one or more turnover pools. Herein, the Cerling *et al.* (2007) model will be referred to as the *two-compartment time based model*:

$$\delta = \delta_f - (\delta_f - \delta_i)(\sum_i p_i\, e^{-\lambda_i t})$$

where p_i = proportion of turnover pool i ($\sum p_i = 1$) and λ_i = turnover rate of pool i. Martinez del Rio and Anderson-Sprecher (2008) suggested using the RPV method described in detail by Cerling *et al.* (2007) to make an initial visual assessment of the number of compartments and using a non-linear fitting algorithm to estimate the parameters. The Levenberg-Marquard algorithm in the graphing and statistical software R (v. 3.0.2, The R Foundation for Statistical Computing) was used to fit the models to the data. An average turnover rate for the two-compartment was then calculated using:

$$\lambda_{mean} = p_1\lambda_1 + p_2\lambda_2$$

where p_1 and p_2 represent the proportions of the two turnover pools, and λ_1 and λ_2 represent the turnover rates associated to those turnover pools. Standard error (SE) of this mean turnover was calculated using the methods described in Martinez del Rio and Anderson-Sprecher (2008).

To compare laboratory turnover rates to field turnover rates, the method from MacAvoy *et al.* (2001) was used:

$$y = x\left(\frac{[Fa - Fb]}{[La - Lb]}\right)$$

where *y* = the maximum expected field turnover rate, *x* = turnover rate measured in the laboratory, *Fa–Fb* = the difference between initial and final isotope values for the field fish tissues and, *La–Lb* = the difference between the initial and final isotope values of the laboratory fish.

2.3.5 Statistical Analysis

The Akaike's Information Criterion corrected for small sample sizes (AIC_c) was used to select the best model to represent the data. The AIC method evaluates the amount of information lost by fitting a model to represent the data. The AIC_c values for each respective model (i.e., AIC_i) are then compared to the model with the lowest AIC_c

value (i.e., AIC_{min}), $\Delta AIC_c = AIC_i - AIC_{min}$. If the ΔAIC_c is ≤ 2 the simpler model is chosen, if the ΔAIC_c is > 2 then the model with the most support (lowest AIC value) is chosen (Burnham and Anderson 2002). The AIC_c values were calculated using:

$$\mathrm{AIC_c} = n\left(1 + ln\left(\frac{2\pi \times RSS}{n}\right)\right) + 2K + \frac{2K(K+1)}{n-K-1}$$

where K is the number of parameters estimated, n is the sample size and RSS is the residual sum of squares from the modelled nonlinear regression. Two-sample t-tests were used to compare if there were statistical differences between the turnover rates and TDFs estimated from the models fitted to the $\delta^{34}S$ values from the laboratory experiment (Motulsky and Christopoulos 2003).

One-sample t-tests were used to determine if the $\delta^{34}S$ turnover rate was due to growth or metabolism and growth (i.e., if c was different from -1). A Kruskal-Wallis test was used to determine if the stocked brook trout were growing over time. A two-way ANOVA was used to determine if the condition factor (CF= (weight(g)*10^5)/ fork length(cm)^3) of the wild and stocked brook trout changed over time; time (7 levels), type (wild and stocked, 2 levels) were fixed factors. A two-way ANOVA was used to assess if there were any differences in the $\delta^{34}S$ values between and within the sampled benthic macroinvertebrate species over time; time (7 levels) and macroinvertebrate species (4 levels) were fixed factors. A two-way ANOVA was used to determine if, over time, there were any difference between liver, muscle and caudal fin tissue of wild brook trout and their assumed diet; tissues including diet (4 levels) and time (7 levels) were fixed factors. Two-sample t-tests were used to determine if there were differences between the $\Delta\delta^{34}S$ values of the wild brook trout tissues. The assumptions of homogeneity of variance and normality of residuals were checked (using Levene's test and normality probability plots, respectively) and met. Statistical analyses were performed in R (v. 3.0.2, The R Foundation for Statistical Computing).

2.4 Results

2.4.1 Laboratory Experiment

2.4.1.1 Growth

Brook trout in the laboratory diet shift experiment grew throughout the experiment. Just prior to the diet shift, the average fish weight was 88.2±23.0g (±SD, n = 20) and on the last sampling event, day 326, average fish weight was 613.1±92.9g (n=19). By the end of the diet shift, all sampled fish had at least quadrupled in weight (Fig. 1). The mean specific growth rate for the duration of the laboratory experiment was 1.0±0.7% body weight gain•day^{-1} (±SD, n = 320, Fig. 2). Specific growth rates varied between individuals across time and ranged from -1.5 to 4.6% body weight gain•day^{-1}. Only seven of the sixty sampled fish experienced negative specific growth rates, or weight loss, and these occurred during the first four days of the diet shift. The specific growth rates of sampled fish and the variability of specific growth rates within a sampling event decreased with time (Fig. 2).

2.4.1.2 Stable Isotope Data

Variable rates of isotopic turnover following a shift to an isotopically distinct diet were obvious for all four tissues sampled throughout the laboratory study (Fig. 4

and 5). The order of fastest to slowest $\delta^{34}S$ turnover was liver, caudal fin, blood and muscle tissue for the growth and time based models. Liver, blood and caudal fin tissue reached a steady state with the diet in terms of both relative growth and time (Fig. 4 and 5). Muscle tissue did not reach a steady state with the diet for either the growth or time based models. The $\delta^{34}S$ values for the laboratory diet and for the tissues on day 0 (pre-diet shift) and day 326 (end of diet shift) are provided in Table 2.

The AIC_c values calculated for the one- and two-compartment time based models show that the two-compartment model best fits the data for all tissues with the exception of caudal fin tissue, in which case the one-compartment model was the best and simplest fit (Table 6). The time based turnover rates and $\Delta\delta^{34}S$ values estimated from the models that were determined to best fit the data according to the AIC_c were used in the interpretation of the results. It should be noted that the estimates from the one-compartment time based model (for all tissue turnover rates and $\Delta\delta^{34}S$ values) were higher significantly different than estimates from the two-compartment time based model (*t*-test, $t_{619 \text{ to } 633} = 3.3$ to 11.3, $p < 0.001$), with the exception of the turnover rate and $\Delta\delta^{34}S$ for caudal fin ($t_{628,\,628} = 0.1, 0.7$, $p > 0.5$) (Tables 4 and 7). Two-compartment models typically estimate lower turnover rates and smaller $\Delta\delta^{34}S$ values (Carleton *et al.* 2008).

2.4.1.3 Tissue Turnover Rates

The growth-based (Fry and Arnold 1982) turnover constants predicted for liver, caudal fin, blood and muscle suggested that the contributions from metabolism to $\delta^{34}S$ turnover varied for each tissue (Table 5). Liver, caudal fin, and blood were significantly different from -1 (t-test, $t_{313\text{ to }320} = 9.7$ to 18.3, $p < 0.001$), indicating there were contributions from metabolic isotope turnover to these tissues. The turnover constant, *c*, for muscle did not differ statistically from -1 ($t_{317} = 0.175$, $p = 0.861$), suggesting that isotopic turnover was due to growth and not metabolism. In terms of relative growth, laboratory brook trout reached 50% turnover in liver, caudal fin, blood and muscle tissue after growing 1.2, 1.3, 1.5, and 2.2 times their initial size, respectively. Brook trout reached 90% turnover in liver, caudal fin, blood and muscle tissue after growing 2.1, 2.5, 3.9, and 12.9 times their initial size, respectively. The number of days to reach 50% and 90% turnover based on the growth-based model for liver, caudal fin, blood and muscle tissue were 11, 15, 24, 56 and 50, 67, 131, 617 days, respectively.

In the laboratory experiment and based on the best time-based model (Hesslein *et al*. 1993, Cerling *et al.* 2007; Martinez del Rio and Anderson-Sprecher 2008), the time-based turnover rates predicted for all tissues suggested that the contributions from metabolism to $\delta^{34}S$ turnover varied for each tissue (Table 6). In terms of days after the diet shift, laboratory brook trout reached 50% turnover in liver, caudal fin, blood and muscle 19, 17, 46, and 63 days, respectively. Liver, caudal fin, blood and muscle reached 90% turnover at 62, 58, 153, and 210 days, respectively. The liver turnover rate estimated by the two-compartment model was significantly different (i.e. faster) than that for muscle and blood ($t_{627\text{ to }623} = 36.8$ to 34.3, $p < 0.001$). The caudal fin tissue turnover rate estimated by the one-compartment model was significantly different (i.e., faster) from muscle and blood turnover rates estimated by the two-compartment model ($t_{628,\ 624} = 9.5, 8.2$, $p < 0.001$). Muscle and blood turnover rates both best estimated by the two-compartment model were significantly different from each other ($t_{620} = 6.2$, $p <$

0.001). Liver and caudal fin tissue turnover rates estimated from the two-compartment model and one-compartment model, respectively, were not significantly different from each other ($t_{631} = 1.0$, $p = 0.324$).

2.4.1.4 Trophic Discrimination Factor ($\Delta\delta^{34}S$)

The average (± SD) $\delta^{34}S$ value measured for the laboratory pellet diet during the experiment was 5.8±0.3‰. The estimated $\Delta\delta^{34}S$ values (i.e. $\delta^{34}S$ consumer - $\delta^{34}S$ diet) ranged from 1.0 to 1.5‰ for liver, caudal fin and blood for both the growth and time-based models. The $\Delta\delta^{34}S$ values predicted from the time-based model were not significantly different from the $\Delta\delta^{34}S$ values predicted from the growth-based models ($t_{618 \text{ to } 630} = 0.4$ to 1.8 $p > 0.05$) for liver, caudal fin, and blood tissue; however, there was a significant difference for muscle tissue ($t_{626} = 3.2$, $p = 0.001$; Table 4). The $\Delta\delta^{34}S$ values estimated for muscle by the growth and time-based model varied the most with a difference of 1.6‰ between the estimates (Table 4).

2.4.2 Field Experiment

2.4.2.1 Growth and Condition

Of the three size-classes of stocked brook trout released in Otter Brook, only two size- classes were recaptured: the 11.0 to 14.9cm size-class and the 15.0 to 22.2cm size-class. We can infer from the fork length of the recaptured brook trout stocked into Otter Brook for the field experiment that there was no growth over the 145 days of the study (Fig. 3). When comparing average length of brook trout at each sampling event to the initial average stocking length there were no statistically significant changes in size (Kruskal-Wallis test, chi squared = 1.12, df = 5 $p = 0.952$). The condition factor of the brook trout before, and after, stocking was measured as a quantitative assessment of the general condition of fish throughout the field study. The condition factor for salmonids typically falls within the range of 0.8 to 2.0 (Barnham and Baxter 1998). Prior to stocking, the average condition factor of the brook trout was 1.04±0.13 (SD, n = 1777). The average condition factor of the recaptured stocked brook trout over the entire experiment was 0.98±0.09 (SD, n = 59), and the average condition factor of the wild brook trout sampled throughout the field study was 1.02±0.15 (SD, n =23). There was no significant difference between the condition factors for the stocked and wild brook trout throughout the field diet shift experiment ($F = 0.760$, $df = 5, 58$, $p = 0.582$).

2.4.2.2 Stable Isotope Data

The $\delta^{34}S$ field turnover rates for brook trout tissues were estimated using only the one-compartment time based model, because the preliminary visual assessment (method described in Cerling *et al.* 2007) showed that there was only one isotope turnover pool. Of the 79 recaptured stocked brook trout, 20 (25%) had lower condition factors than the lowest condition factor of the wild brook trout and were therefore excluded from analysis because it was inferred that these fish were not well adapted to their new habitat and were not feeding at a similar rate to wild brook trout. There was no significant difference in the turnover rates between the two size-classes of stocked brook trout ($t_{47, 47} = 0.9, 0.9$, $p > 0.1$), therefore data were pooled and no distinction was made between the size classes for data analysis (Fig. 6).

2.4.2.3 Tissue Turnover Rates

The turnover rates determined from the field study for liver and caudal fin were 0.014±0.007 and 0.048±0.013‰ • day^{-1}, respectively, and were significantly different (t_{100} = 16.4, p = 0.023). Unlike liver and caudal fin field δ^{34}S values that were fitted with an exponential model, muscle was fitted with a linear equation. Muscle did not turnover from day 0 to 145 (Fig. 6). Based on the metabolic turnover rate (*m*) determined for liver and caudal fin tissue in the laboratory study (Table 6), the expected *m* for both of these tissues in the field study would be 0.012‰ • day^{-1} (based on the equation described by MacAvoy *et al.* (2001) that takes into account the difference between initial and final δ^{34}S values for the laboratory and field experiment). The observed *m* for liver in the field study was 0.014‰ • day^{-1}, similar to expected metabolic turnover rate of 0.012‰ • day^{-1} calculated from the laboratory result. The observed caudal fin turnover for fin clip tissue was 0.048‰ • day^{-1}, which was considerably greater than the expected turnover rate of 0.012‰ • day^{-1} calculated from the laboratory data.

2.4.2.4 Trophic Discrimination Factor ($\Delta\delta^{34}$S)

The stocked brook trout from the field diet shift did not reach isotopic steady state with their presumed wild diet, therefore a $\Delta\delta^{34}$S could not be estimated for the stocked brook trout. There was no significant difference in δ^{34}S values between wild brook trout liver, caudal fin and muscle and benthic macroinvertebrates throughout the duration of the field study (F = 0.629, df = 3, 100, p = 0.598; Fig. 6). There were no significant differences in the δ^{34}S values within and between macroinvertebrate functional feeding groups over time (F = 0.615, df = 18, 17, p = 0.842). Therefore, the

$\delta^{34}S$ values of the benthic macroinvertebrate functional feeding groups sampled throughout the field experiment were grouped into one averaged $\delta^{34}S$ value for diet for both stocked and wild brook trout (Table 3). The $\Delta\delta^{34}S$ values determined for liver, muscle and caudal fin tissue from the wild were not statistically different from zero ($t_{39\text{ to }41}$ = 0.308 to 1.257, $p > 0.1$; Table 4).

2.5 Discussion

To our knowledge, this is the first study to show significant differences in $\delta^{34}S$ turnover rates and varying contributions of metabolism to $\delta^{34}S$ turnover in fish tissues, as well as to report the trophic discrimination factor ($\Delta\delta^{34}S$) for $\delta^{34}S$ in four fish tissues: liver, caudal fin, blood and muscle. This study is also the first to compare $\delta^{34}S$ turnover rates and $\Delta\delta^{34}S$ values in multiple tissues in both wild and laboratory settings.

2.5.1 Tissue Turnover Rates

Tissue-specific turnover rates for $\delta^{34}S$ were observed in our study; this is in contrast to previous research that did not conclusively show differences in $\delta^{34}S$ turnover in different tissues (Hesslein *et al.* 1993; MacAvoy *et al*. 2001). MacAvoy *et al.* (2001) may not have detected tissue specific turnover rates due to the variability in the initial $\delta^{34}S$ values among the channel catfish (*Ictalurus punctatus*), as well as the variability of the $\delta^{34}S$ values of their diet. Hesslein *et al.* (1993) observed that the sulphur turnover was driven by tissue addition (i.e., growth) in liver and muscle tissue of broad whitefish (*Coregonus nasus*) and tissue replacement (i.e., metabolism) did not result in tissue-specific turnover rates. Unlike Hesslein *et al.* (1993), our findings are due to the varying contributions of metabolism to isotopic turnover in each tissue. Protein metabolism in splanchnic tissues such as liver is greater than in structural tissues such as muscle (Tieszen *et al*. 1983; Smith 1981; de la Higuera *et al*. 1999; Wolf *et al*. 2009), and other studies on fish have found significant differences in $\delta^{13}C$ and $\delta^{15}N$ turnover between tissues (Suzuki *et al.* 2005; Logan *et al.* 2006; Buchheister and Latour 2010; Heady and Moore 2013).

Our results from both the growth- and time-based models indicated that $\delta^{34}S$ turnover in muscle was only due to growth, and furthermore that the contribution of metabolism to $\delta^{34}S$ turnover approached zero (Tables 5 and 6). The negative value of the metabolic component (an artifact of the model) represents that metabolism was not a contributing factor to the overall turnover rate. In reality, there is potential for protein replacement (i.e., metabolism) to contribute sulphur turnover in muscle tissue, but in our case the effect of metabolism was considered to be negligible relative to the effect of growth on sulphur turnover in muscle tissue. Other studies have found very little contribution of metabolism to carbon and nitrogen turnover in muscle tissue in summer flounder (*Paralichthys dentatus*) and rainbow trout (*Oncorhynchus mykiss*) (Buchheister and Latour 2010; Heady and Moore 2013). Buchheister and Latour (2010) also observed a *negative* metabolic contribution to carbon turnover in summer flounder muscle tissue when using a growth-based model.

The caudal fin turnover rates derived from the lab and field experiments were likely affected by elevated levels of fin erosion. Caudal fin erosion, that often occurs when being held in artificial systems (tanks), leads to constant repair and tissue addition. The constant tissue repair and addition likely lead to the atypically high $\delta^{34}S$ turnover rate observed in our study. In the field experiment, hatchery-reared brook trout had eroded caudal fins prior to being stocked. Recaptured brook trout had full caudal fin regrowth by sampling day 29 (03 July 2012), at which time caudal fin tissue turnover slowed down and ceased by the next sampling event on day 47. From sampling day 29 to day 145 (03 July to 27 October 2012), caudal fin $\delta^{34}S$ turnover resembled that of muscle, a tissue where turnover was more affected by growth than metabolism in the laboratory study. If caudal fin turnover in a natural setting matched that of liver, we would expect that these tissues would reach the same $\delta^{34}S$ on day 359 (29 May 2013) of the field study, which they did not. Heady and Moore (2013) found that fin tissue turnover rates were comparable to liver for nitrogen, which is similar to what we found in the laboratory experiment in terms of sulphur turnover. In terms of the differences of turnover rates between tissues, the results from Suzuki *et al.* (2005) on Japanese temperate bass (*Lateolabrax japonicas*) carbon and nitrogen turnover were similar to our study's finding for sulphur turnover in brook trout tissues in the field, with the exception of caudal fin tissue. Suzuki *et al.* (2005) found that the carbon and nitrogen turnover rates of fin tissue for bass juveniles were almost identical to the turnover rates of muscle tissue, whilst the turnover rates for liver tissue were more rapid.

Fin tissue is often sampled in field studies because it is non-lethal. The caudal fin is composed of bony rays and skin. A considerable amount of fin tissue (5 mg dried tissue) is required for sulphur stable isotope analysis, typically consisting of a combination of skin and fin rays. Fin rays are comprised of collagen, a structural tissue associated with slow turnover rates (Carleton *et al.* 2008). Reich *et al.* (2008) found that the stable isotope turnover rates of skin in fast growing juvenile ectotherms (loggerhead sea turtle, *Caretta* caretta) were similar to whole blood for nitrogen and nearly double for carbon. If fin ray and skin tissues are likely to have slower turnover rates relative to more metabolically active tissues such as liver, it would be expected that the caudal fin tissue would have a slow turnover rate comparable to a slow turnover tissue such as muscle, as seen in Japanese temperate bass (Suzuki *et al.* 2005).

Whole blood, which was sampled in the laboratory experiment, has different components: plasma, platelets, red and white blood cells. Two of these components, plasma and red blood cells, are known to have different turnover rates in terms of carbon and nitrogen in a variety of taxa (Hilderbrand *et al.* 1996; Phillips and Eldrige 2006; Reich *et al.* 2008; Klaassen *et al.* 2010; Caut *et al.* 2011). The $\delta^{34}S$ turnover rate of whole blood in our laboratory experiment reflects the combination of (1) a tissue with a relatively slow turnover rate (i.e., red blood cells) that is comparable to C and N turnover rates in muscle of other fish species, and (2) a tissue with a relatively rapid turnover rate (i.e., plasma) that is comparable to C and N turnover rates in liver of other fish species. In rainbow trout, plasma $\delta^{15}N$ turnover rate (0.071‰ • day^{-1}) closely resembled $\delta^{15}N$ turnover rate of liver (0.062‰ • day^{-1}), while red blood cells $\delta^{15}N$ turnover rate (0.027‰ • day^{-1}) resembled $\delta^{15}N$ turnover rate of muscle (0.026‰ • day^{-1}) (Heady and Moore 2013). Buchheister and Latour (2010) also found similar groupings of comparable turnover rates in summer flounder. The turnover rates of whole blood estimated in our study are greater than muscle and slower than liver as expected since whole blood is mix of tissues with both fast and slow turnover rates.

Unlike the laboratory experiment, growth was likely not a significant factor influencing $\delta^{34}S$ in our field experiment, where average fork length did not change significantly over the course of the sampling period. The results from the laboratory study indicated that $\delta^{34}S$ turnover in muscle was influenced by growth alone therefore in the absence of growth we would expect there to be no $\delta^{34}S$ turnover in muscle. During the field experiment, the sulphur in stocked brook trout muscle tissue did not turnover; from this we can concluded that there was no significant tissue addition (growth) during the 145 days that the stocked brook trout were sampled from the stream. The lack of growth during the field experiment was expected since the brook trout were stocked and sampled during a time of year when growth rate is slow (Carlson *et al.* 2007; Xu *et al.* 2010). The high initial stocking density (0.6 brook trout • m^2) could have also affected growth. Utz and Hartman (2009) noted a reduction of growth rates of brook trout with an increase in the population's density, likely due to prey availability. If during the field experiment growth did not contribute to sulphur turnover of the stocked brook trout tissues, then the $\delta^{34}S$ turnover of the liver and caudal fin tissues must be due to metabolism. Variability in tissue turnover rates and relative contribution of metabolism and/or somatic growth are critical for evaluating the incorporation of dietary isotopes.

2.5.2 Trophic Discrimination Factors ($\Delta\delta^{34}S$)

In general, the $\Delta\delta^{34}S$ values estimated in our laboratory study did not differ significantly between the growth based and time based models, or between different tissues. The only exception was for muscle estimated by the two-compartment time based model, where the average $\delta^{34}S$ of muscle tissue on the last sampling event did not reach steady state. These models were limited by the available data to predict a steady state value, which in turn was used to determine the $\Delta\delta^{34}S$. Since the laboratory diet shift study was not long enough for muscle to reach a steady state, the predicted value was greater than expected and, therefore, the $\Delta\delta^{34}S$ (for muscle) estimated by the two-compartment time based model was also greater than expected. Brook trout with higher growth rates were much closer to reaching the $\delta^{34}S$ steady state. The growth model more closely characterized the true $\Delta\delta^{34}S$ of the muscle tissue than the time model. This is because the time model only represents the time post diet shift and does not take into account the relative growth of the brook trout during the laboratory experiment. Given that muscle tissue did not reach steady state with the diet, we are not confident in the validity of the $\Delta\delta^{34}S$ values for muscle tissue estimated from the growth and time models.

Hesslein *et al.* (1993) noted a $\Delta\delta^{34}S$ that differed from zero and suggested that this was possibly due to differential assimilation of the pelletized diet components. It is possible that the $\Delta\delta^{34}S$ values we measured in the tissues from the laboratory study were also due to preferential assimilation of certain components of the pelletized diet. The $\delta^{34}S$ value of tilapia meal, a source for sulphur bearing amino acids in the pelletized diet, is similar to the $\delta^{34}S$ of the brook trout tissues at steady state (Table 1). Plant material made up 25% of the pelletized diet composition. Plant tissues contain inorganic sulphur, mostly in the form of sulphate that is not biologically available to animals until it is biosynthesized by plants into methionine and cysteine (Krouse *et al.* 1991; Saito 2004). The pelletized diet likely contains inorganic sulphur from plant material; therefore the $\delta^{34}S$ of the diet would not represent only the $\delta^{34}S$ of biologically available sulphur to the brook trout. The $\Delta\delta^{34}S$ observed in the laboratory study is a result of the influence of inorganic sulphur on the $\delta^{34}S$ value of the diet. In a study by Newsom *et al.* (2011), it appeared that the gastrointestinal microbiota provided their hosts, tilapia, with essential amino acids if the dietary sources did not provide sufficient quantities of essential amino acids. The pelletized diet used in the laboratory study was developed for salmonids and was assumed to meet all the dietary requirements of brook trout, but it is possible that the diet did not provide sufficient quantities of methionine. A diet depleted in methionine but containing sulphate, which would be available to gastrointestinal microbiota for methionine biosynthesis, could also explain the $\Delta\delta^{34}S$ observed in the brook trout tissues.

2.5.3 Conclusion

Studies by Peterson *et al.* 1985, Hesslein *et al.* 1993, MacAvoy *et al.* 2001 and Barnes and Jennings 2007 have quantified the turnover rates and $\Delta\delta^{34}S$ for $\delta^{34}S$ in fishes. Our work has expanded on these findings by demonstrating how tissue specific turnover rates vary between tissues, as well as how turnover rates and $\Delta\delta^{34}S$ values can vary between field and laboratory studies. The tissue specific turnover rates estimated in the laboratory study due to the varying contributions of metabolism and growth to turnover resulted in different tissues recording changes in diet on different time scales. This observation was obvious in the field experiment where, in the absence of growth, muscle $\delta^{34}S$ did not turnover but there was turnover in liver due to metabolism. The $\Delta\delta^{34}S$ results from the laboratory ($\Delta\delta^{34}S$ ~1‰) and the field ($\Delta\delta^{34}S$ ~0‰) experiments highlight the potential for sulphur as a food source indicator, as its range for its $\Delta\delta^{34}S$ values is comparable to that of carbon ($\Delta\delta^{13}C$ typically 0-1‰). We cannot say with certainty whether the presence of inorganic sulphur in the diet, the preferential assimilation of dietary components, or the contributions of essential amino acid by gastrointestinal microbiota played a role in the $\Delta\delta^{34}S$ values observed in the laboratory study. The $\Delta\delta^{34}S$ values estimated from wild brook trout tissues and benthic macroinvertebrates, which averaged near 0‰, were as expected. Sulphur isotope turnover rates and trophic discrimination factors are essential for the application of sulphur stable isotope analysis to ecological studies. Understanding what affects the turnover rates and the trophic fractionation of sulphur will be important for the future application of sulphur stable isotope analysis.

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Table 1: Percent contribution by mass and $\delta^{34}S$ values of dietary components in the customized pelletized diet used for the laboratory diet shift experiment. To obtain a diet with a depleted $\delta^{34}S$ value relative to the initial diet of the brook trout, a variation on the salmonid pelletized diet developed by Lall and Castell (unpublished) of Fisheries and Oceans Canada was produced. The original pelletized diet was produced with herring fishmeal, which was replaced with that of a freshwater species, tilapia (*Oreochromis niloticus*).

Ingredients	Contribution (%)	$\delta^{34}S$ (‰)
Tilapia Muscle	50.0	7.8
Wheat Middlings	12.0	-
Dextrin	10.0	-
Herring Oil	8.0	-
Soybean Meal (48% CP)	6.0	2.9
Wheat Gluten	5.0	-6.1
Vitamin/Mineral Mix (mod. B-T)	4.0	2.5
Soy Lecithin	2.0	-
Sodium Alginate	1.3	-
Choline Chloride	0.7	-
CMC (carboxymethylcellulose)	1.0	-

Table 2: Initial and final mean $\delta^{34}S$ values ± SD for brook trout (*Salvelinus fontinalis*) tissues from the laboratory diet shift experiment at UNB. The $\delta^{34}S$ value for the pellet diet is also shown for reference. Hatchery reared age 2+ brook trout were fed a diet with a low $\delta^{34}S$ value, relative to their previous diet that had a high $\delta^{34}S$ value. The duration of the diet shift experiment was 326 days.

	Diet (n)	Liver (n)	Caudal Fin (n)	Blood (n)	Muscle (n)
Day 0	5.8±0.3 (40)	17.4±0.4 (20)	17.1±0.4 (20)	17.5±0.4 (20)	16.7±0.2 (18)
Day 326		6.9±0.3 (18)	7.3±0.5 (18)	7.4±0.2 (18)	8.1±0.4 (18)

Table 3: The mean $\delta^{34}S$ values (± SD) for the diet, wild brook trout (*Salvelinus fontinalis)*, stocked brook trout at initial (day 0) and final (day 145) sampling events of the field diet shift study. As well, the $\delta^{34}S$ value for a single stocked brook trout sampled during spring 2013, 359 days after stocking, is indicated. During the spring of 2012, we stocked hatchery-reared brook trout into Otter Brook (with natural barriers to minimize movement) and periodically sampled fish to measure isotopic tissue turnover. Because trout reared on a pellet diet (marine origin), it was possible to trace their diet shift to freshwater food.

	Diet	n	Diet	Liver	Caudal Fin	Muscle	n
Wild Day 0 to 359	5.7±0.8	48	5.7±0.8	5.7±0.8	5.4±0.9	5.3±0.9	22
Stocked Day 0		48	5.7±0.8	11.4±0.4	11.7±0.3	11.5±0.3	6
Stocked Day 145		48	5.7±0.8	9.8±0.9	9.9±0.6	11.7±0.3	8
Stocked Day 359		48	5.7±0.8	6.9	7.7	8.7	1

Table 4: Trophic discrimination factors ($\Delta\delta^{34}S$) for brook trout (*Salvelinus fontinalis*) tissues estimated from both the laboratory and field diet shift experiments. The laboratory $\Delta\delta^{34}S$ estimates were predicted using (1) the growth based model described in Fry and Arnold (1982), (2) one-compartment time based model described in Hesslein *et al.* (1993) and (3) the two-compartment model described in Cerling *et al.* (2007) and Martinez del Rio and Anderson-Sprecher (2008). The $\Delta\delta^{34}S$ values for the field study were calculated from the difference between the mean $\delta^{34}S$ value of wild brook trout and the mean $\delta^{34}S$ of benthic macroinvertebrates. The standard error terms (SE) for the $\Delta\delta^{34}S$ values are asymptotic standard error for estimates. Bolded $\Delta\delta^{34}S$ values for the time-based model indicate the values predicted from the models that fit the data best. The AIC_c values used to assess the best fit are indicated in table 6.

Tissue	Laboratory $\Delta\delta^{34}S$ ± SE			Field $\Delta\delta^{34}S$ ± SE
	Growth Based Model	Time Based Model		
		One-Comp.	Two-Comp.	
Liver	1.2±0.1	1.9±0.1	**1.1±0.**2	0.0±1.1
Caudal Fin	1.0±0.2	**1.5±0.1**	1.3±0.2	-0.2±1.2
Blood	1.3±0.2	2.2±0.1	**1.2±0.3**	NA
Muscle	0.6±0.4	2.9±0.1	**2.2±0.3**	-0.3±1.2

Table 5: Growth based laboratory turnover rates of $\delta^{34}S$ values (*c*) predicted for different tissues of brook trout (*Salvelinus fontinalis*) using the relative growth model described in Fry and Arnold (1982). Estimates of the proportion of sulphur turnover rates attributed to metabolism (P_m) and growth (P_k) in liver, muscle, blood and caudal fin tissues are also provided. The standard error terms (SE) for *c* values are asymptotic standard error for estimates.

Tissue	*c* value ± SE	P_m	P_k
Liver	-3.2±0.1	0.61	0.39
Caudal Fin	-2.5±0.1	0.52	0.48
Blood	-1.7±0.1	0.33	0.67
Muscle	-0.9±0.1	-0.07	1.07

Table 6: The estimated time based laboratory turnover rates of $\delta^{34}S$ for brook trout (*Salvelinus fontinalis*) tissues for tissue replacement and addition (*m*+*k*), tissue replacement only (*m*), and residency time (τ). The *m*+*k*, *m* and τ values values were predicted using the one-compartment time based model described in Hesslein *et al.* (1993) and the two-compartment model described in Cerling *et al.* (2007) and Martinez del Rio and Anderson-Sprecher (2008). The standard error terms (SE) for the turnover rates are asymptotic standard error for estimates. As well, the proportion of sulphur turnover rates attributed to metabolism (P_m) and growth (P_k) in liver, muscle, blood and caudal fin tissues are indicated. As well, the AIC_c values calculated for the one- and two- compartment time based models are indicated. Bolded values indicate the values predicted from the models that fit the data best.

Tissue	One-Compartment Time Based Model					
	m+*k*±SE	*m*±SE	τ±SE	P_m	P_k	AIC_C
Liver	0.059±0.003	0.046±0.003	17.1±0.8	0.78	0.22	761
Caudal Fin	**0.040±0.002**	**0.027±0.002**	**25.1±1.2**	**0.68**	**0.32**	**977**
Blood	0.024±0.001	0.011±0.001	42.4±1.9	0.46	0.54	726
Muscle	0.013±0.001	-0.001±0.001	74.8±3.2	-0.08	1.08	573
	Two-Compartment Time Based Model					
	m+*k*±SE	*m*±SE	τ±SE	P_m	P_k	AIC_C
Liver	**0.037±0.0004**	**0.024±0.008**	**27.1±5.6**	**0.65**	**0.35**	**873**
Caudal Fin	0.035±0.040	0.022±0.040	28.6±26.1	0.63	0.37	978
Blood	**0.015±0.004**	**0.002±0.004**	**66.7±15.6**	**0.13**	**0.87**	**831**
Muscle	**0.011±0.007**	**-0.002±0.007**	**91.4±52.8**	**-0.18**	**1.18**	**598**

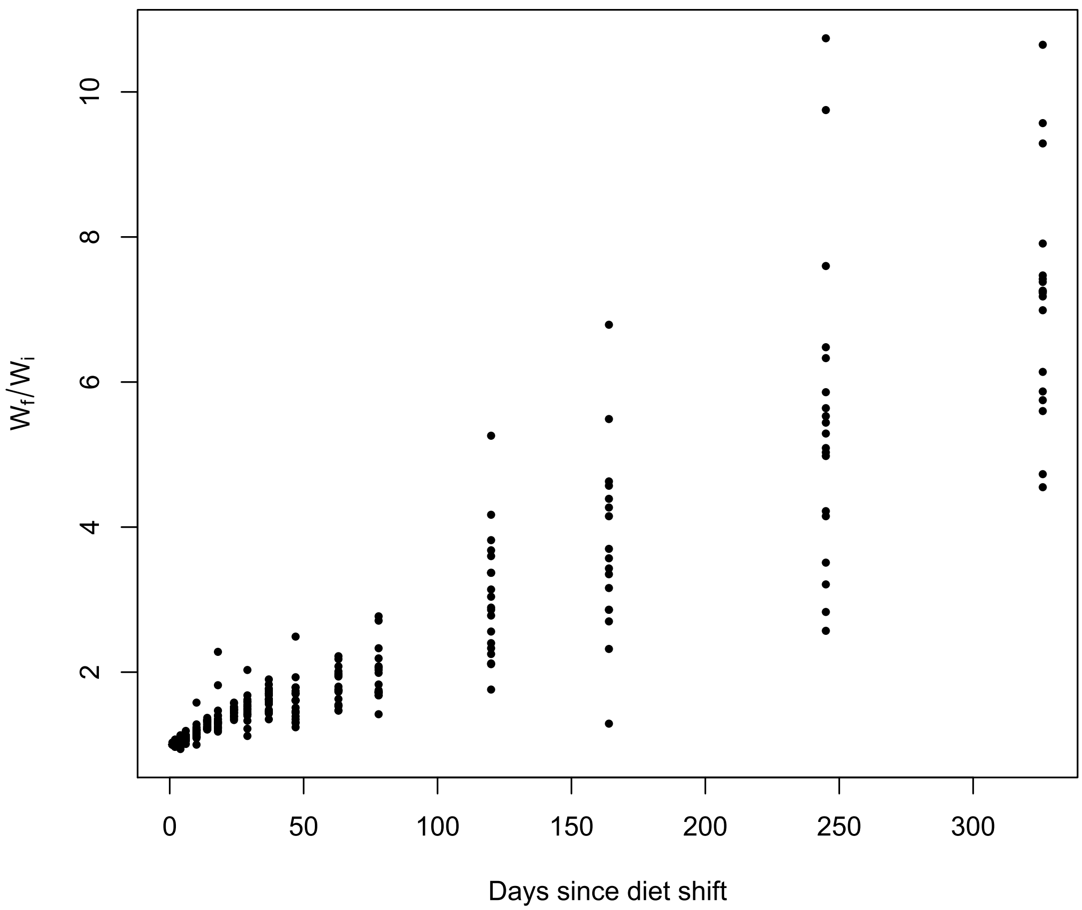

Figure 1: Individual relative growth (W_f/W_i, W_i = initial fish weight (g), W_t = fish weight on the day it was lethally sampled) for laboratory brook trout (*Salvelinus fontinalis*) throughout the laboratory diet shift experiment. The diet shift experiment took place over a period of 326 days.

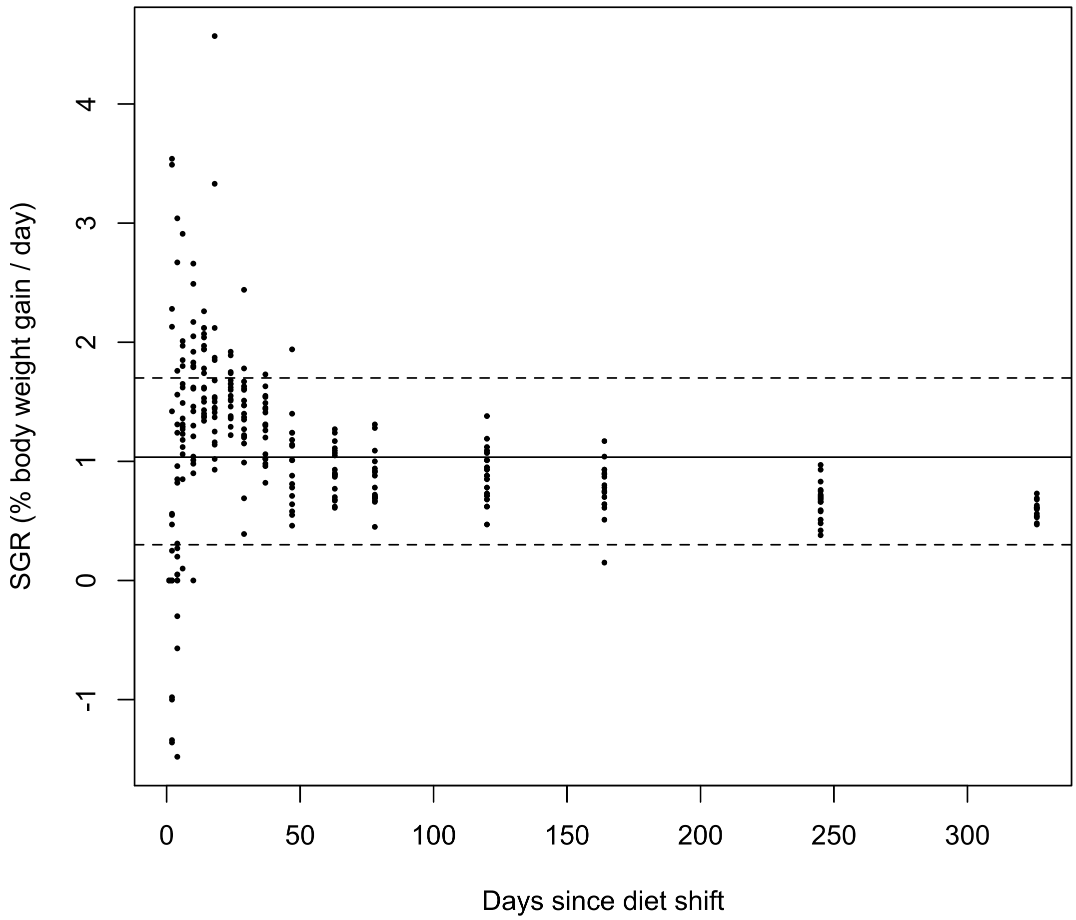

Figure 2: Individual specific growth rates (SGR) for brook trout (*Salvelinus fontinalis*) from the laboratory diet shift experiment. The mean specific growth rate over the course of the experiment (326 days), represented by the solid horizontal line, was 1.0% body weight gain · day^{-1} (the SD about the mean was ± 0.7%, dashed line).

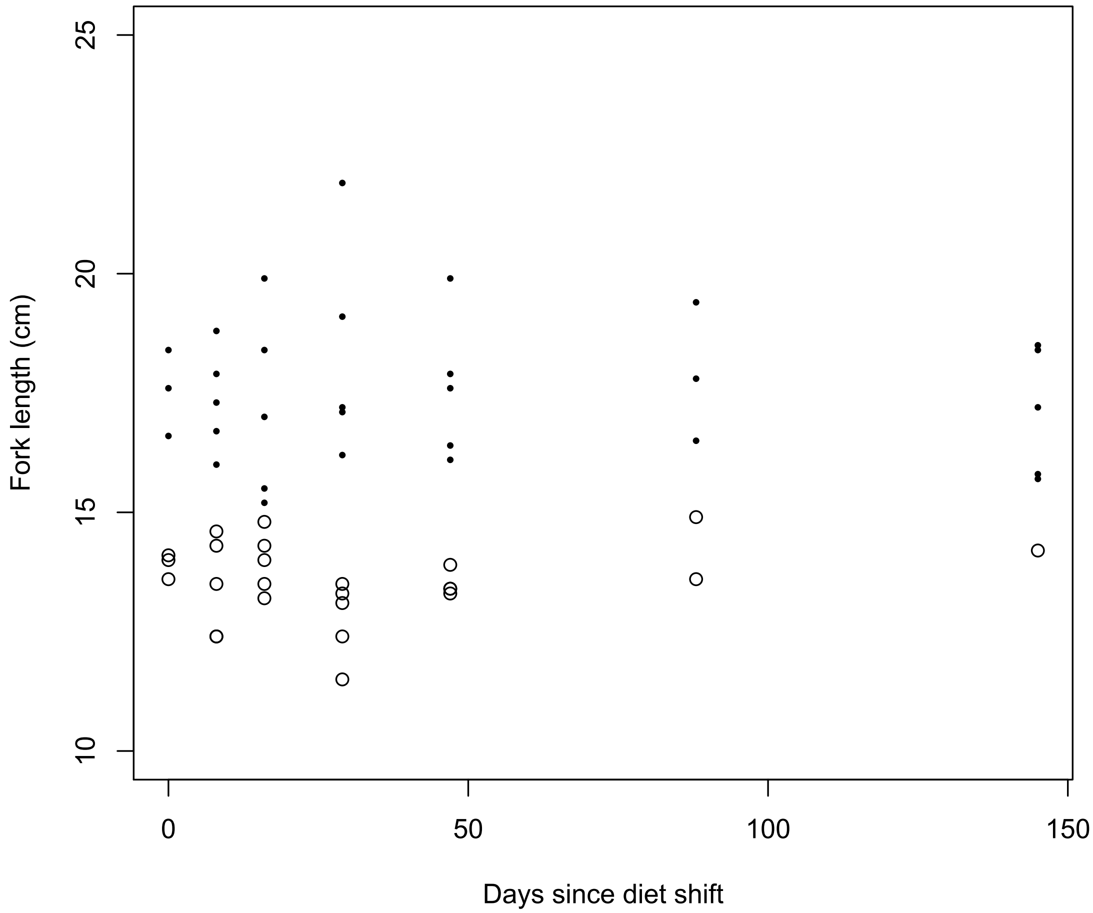

Figure 3: The fork lengths (cm) of recaptured hatchery-reared brook trout (*Salvelinus fontinalis*) stocked into Otter Brook on 04 June 2012 as a diet shift field experiment. Fish were tagged in the anal fin with different coloured visual implant elastomer (VIE) tags to differentiate between size ranges. Two of the three size ranges were recaptured; yellow VIE tagged fish initially ranged from 11.0 to 14.9 cm in fork length (open circles (○)) and red VIE tagged fish initially ranged from 15.0 to 22.2 cm in fork length (solid circles (•)). Day 0 represents 04 June 2012 and the final sampling event (day 147) occurred on 27 October 2012.

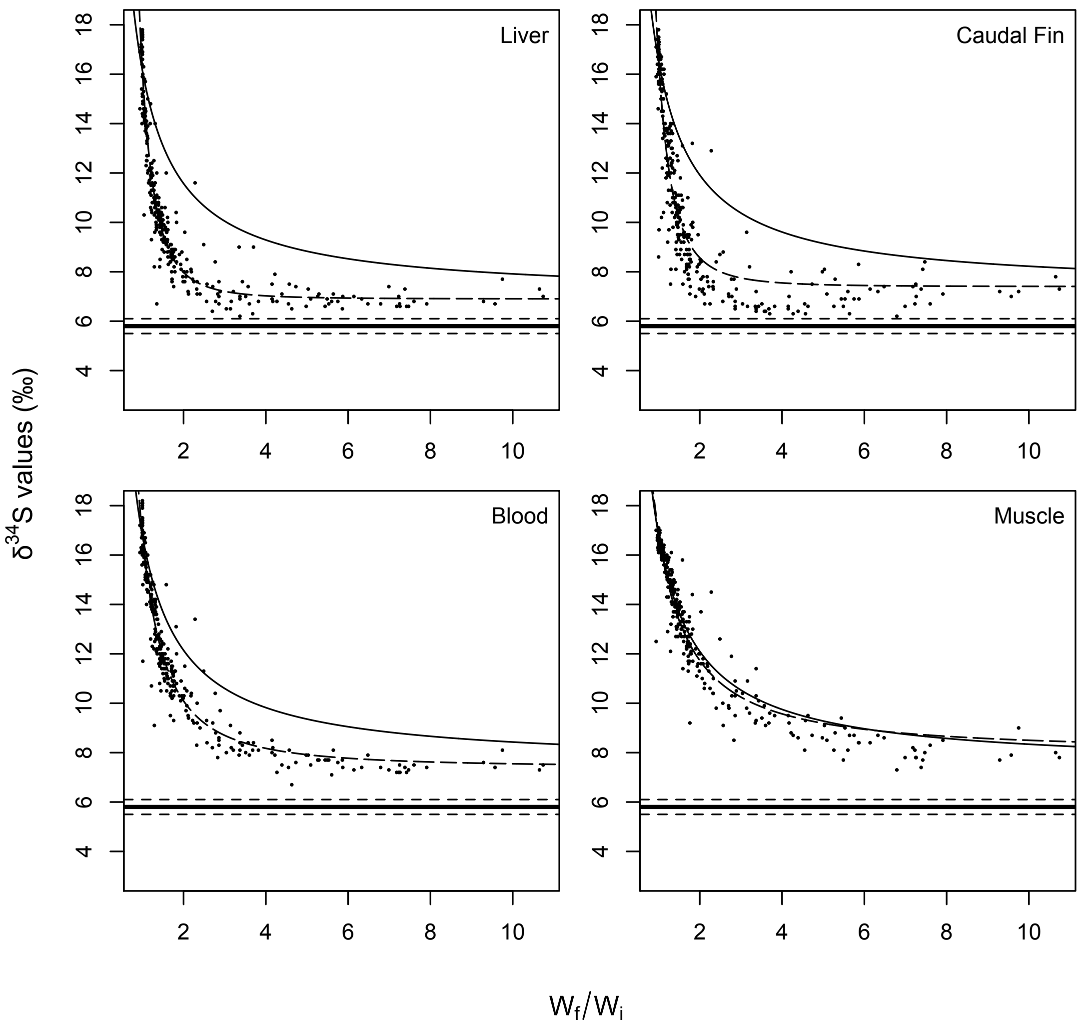

Figure 4: Incorporation of δ^{34}S in liver, caudal fin, blood and muscle in laboratory brook trout (*Salvelinus fontinalis*) as a function of relative growth (W_f/W_i, W_i = initial fish weight (g), W_t = fish weight on the day it was lethally sampled). The dashed line represents the non-linear curve fit that best describes the rate of incorporation. The δ^{34}S incorporation expected from dilution only, *c* = -1, is represented by a solid curve. The horizontal solid line represents the mean δ^{34}S value of the diet (pellet) and the horizontal dashed lines represent the standard deviation of the diet δ^{34}S values.

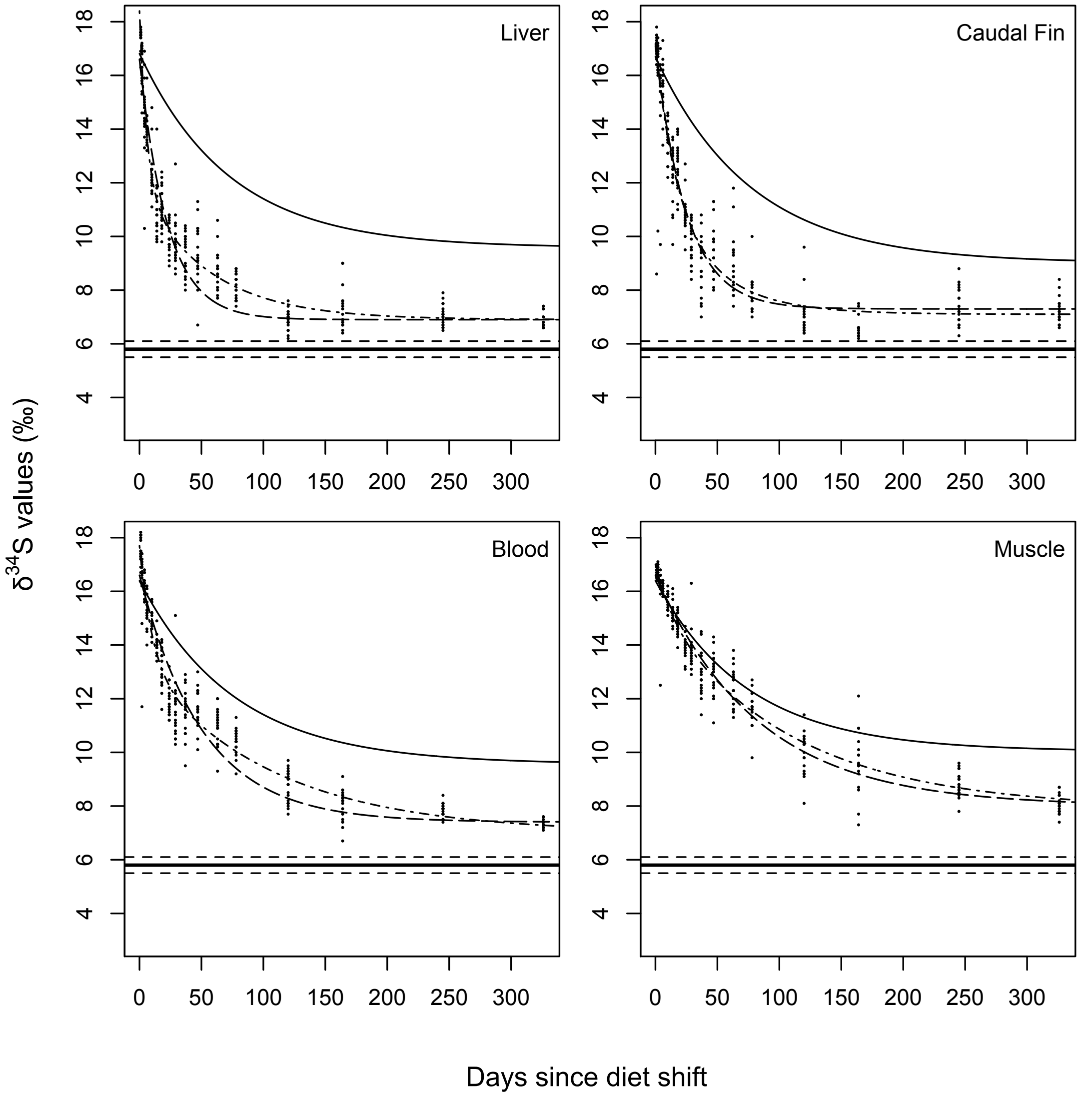

Figure 5: Temporal changes in $\delta^{34}S$ in liver, caudal fin, blood and muscle tissues in laboratory reared brook trout (*Salvelinus fontinalis*) following the diet shift. The dashed line represents a one compartment non-linear curve fit and the dot-dashed line represents a two compartment non-linear curve fit. The solid curve represents the pattern of $\delta^{34}S$ incorporation when only growth is contributing. The horizontal solid line represents the mean diet $\delta^{34}S$ value for the diet and the horizontal dashed lines represent the standard deviation of the diet $\delta^{34}S$ values.

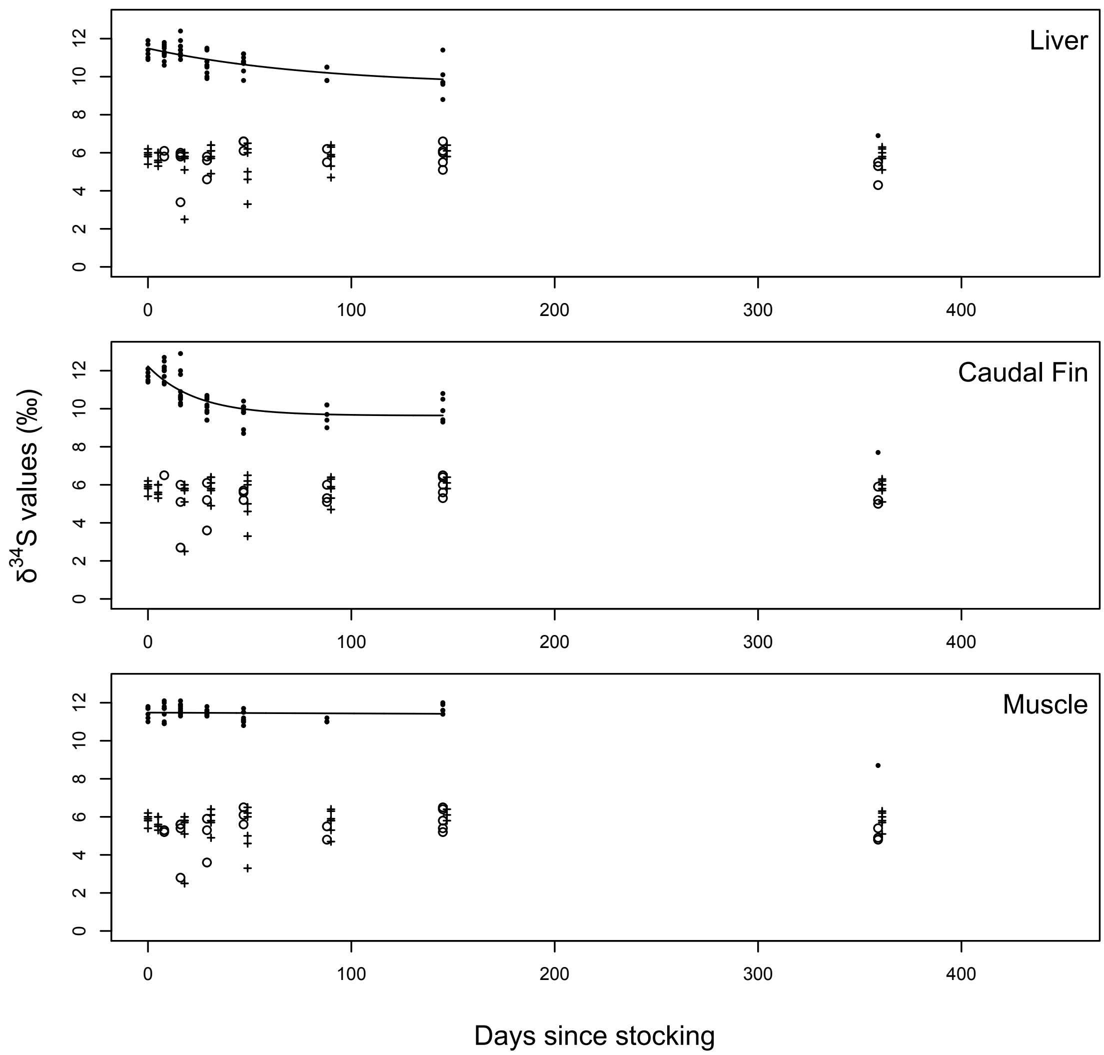

Figure 6: Incorporation of $\delta^{34}S$ in liver, caudal fin, muscle in stocked brook trout (*Salvelinus fontinalis*) (•) through time following the diet shift. Included are the $\delta^{34}S$ values of wild brook trout (○) and benthic macroinvertebrates (+) sampled in Otter Brook. The solid curve represents a non-linear fit to liver and caudal fin $\delta^{34}S$ values from day 0 to 145 and a linear fit to muscle $\delta^{34}S$ values from day 0 to 145. Day 0 represents 04 June 2012 and day 359 represents 29 May 2013.

3.0 General Discussion

The turnover rates and trophic discrimination factors ($\Delta\delta^{34}S$) of sulphur isotope ratios in brook trout tissues reported here are based on a unique study that (1) was performed on several tissues (liver, muscle, blood and caudal fin) and (2) consisted of a laboratory diet shift experiment as well as a field diet shift experiment. The sulphur turnover rates determined in the laboratory diet shift experiment were different for each tissue in terms of contributions of tissue addition (growth) and tissue replacement (metabolism) to sulphur turnover. The turnover rates observed in the field provided some insight on how the turnover rates can vary temporally, i.e., depending on whether the brook trout were growing or not. The $\Delta\delta^{34}S$ values were ~1‰ and ~0‰ for the laboratory and field experiments, respectively.

3.1 Tissue Turnover Rates

The successful application of stable isotope analysis in ecology is reliant on our knowledge of the isotope turnover rates and $\Delta\delta^{34}S$ values of organism's tissues. The laboratory experiment tracked sulphur turnover in brook trout tissues with growth data for each individual brook trout. This was the aim because growth data are necessary to assess the contribution of growth and metabolism to sulphur isotope turnover in different tissues. The relative contributions of growth and metabolism to different tissues helped us understand the sulphur isotope turnover, so that the expected outcome of similar experiments might be modeled. This knowledge is essential to explain results obtained from the tissues of the field experiment and can also benefit fish physiology studies.

Both the laboratory and field diet shifts illustrate sulphur turnover rates are tissue-specific, and the field experiment highlights the potential for temporal variation of the tissue-specific turnover rates. This is important because different tissues will record dietary changes on different timescales, timescales that could vary for a tissue throughout the year. Perga and Gerdeaux (2005) observed the absence of carbon and nitrogen isotope turnover in whitefish (*Coregonus lavertus*) muscle during periods when no growth occurred. Liver on the other hand showed continuous isotope turnover and matched the changes in carbon and nitrogen of their zooplankton diet throughout the year. The difference in isotope turnover rates of different tissues can be used to produce an "isotope clock" by providing information on varying temporal scales (Fry 2006; Phillips and Eldridge 2006; Klaassen *et al*. 2010; Buchheister and Latour 2010; Heady and Moore 2013). By comparing different tissues, it is theoretically possible to determine when the diet shift of fish occurred. The field experiment demonstrates the importance of understanding the roles of metabolism and growth in each tissue and how temporal variation of a fish's physiology have the potential of affecting isotope turnover rates.

3.2 Trophic Discrimination Factors ($\Delta\delta^{34}S$)

Isotopic discrimination for carbon and nitrogen can be explained, in part, by the discrimination associated with protein synthesis from nonessential amino acids. Unlike carbon and nitrogen, *organically-bound* sulphur in organism tissues is only found in two sulphur bearing essential amino acids; methionine and cysteine (Krouse *et al.* 1991; Hobson 2008). Both of these amino acids are acquired via the diet *(essential)* or, in the case of cysteine, can also be biosynthesised from methionine *(conditionally essential)* (Koch 2007; Hobson 2008). The $\Delta\delta^{34}S$ was expected to not differ from zero. There is evidence that consumers assimilate some essential dietary amino acids with minimal or no isotopic fractionation of ^{13}C and ^{15}N; these amino acids are called source amino acids, while other dietary essential amino acids assimilated with trophic discrimination are called trophic amino acids (Howland *et al.* 2003; Popp et al. 2007; Hobson 2008; Hannides et al. 2009; McMahon *et al.* 2010; Bradley *et al.* 2014). Methionine, the only truly essential amino acid containing sulphur, is assimilated with minimal trophic discrimination between fish and zooplankton in terms ^{15}N and the $\Delta\delta^{34}S$ ranges approximately between -0.5‰ to 0‰ (Chikaraishi *et al.* 2009). $\Delta\delta^{34}S$ values from our laboratory study are similar to what Peterson *et al.* (1985) and Hesslein *et al.* (1993) estimated from for brook trout (*Salvelinus fontinalis*) and broad whitefish (*Coregonus nasus*), respectively. Hesslein *et al.* (1993) fed the brook trout a pelletized diet such as we did with the laboratory experiment. Barnes and Jennings (2007) found that the average $\Delta\delta^{34}S$ of European sea bass, *Dicentrarchus labrax*, relative to its diet, was -0.5±0.1‰, a result more comparable to the $\Delta\delta^{34}S$ values estimated from our field experiment. Barnes and Jennings (2007) fed European sea bass what they would prey upon in the wild. Our field experiment allowed the stocked brook trout to consume a natural diet. Diet quantity and quality affect the $\Delta\delta^{34}S$ values for carbon and nitrogen (Post 2002; Gaye-Siessegger *et al.* 2003; McCutchan *et al.* 2003; Robbins *et al.* 2010), and quantity affect the $\Delta\delta^{34}S$ of sulphur (Barnes and Jennings 2007). The role of the quality of the diet may also play an important role in the $\Delta\delta^{34}S$ values for sulphur. The uncertainty with regards to food quality highlights the importance for further information on what affects the $\Delta\delta^{34}S$ in fish tissues; for example, if a low quality diet does not provide sufficient amounts of methionine, will the gastrointestinal microbiota provide methionine to their hosts with a distinct $\delta^{34}S$ from that of the diet?

3.3 Application

The tissue-specific sulphur turnover rates, the low $\Delta\delta^{34}S$ values and the difference between marine and freshwater $\delta^{34}S$ values suggest sulphur could be a useful tracer for migratory fish movements between freshwater and marine systems. For example, in the case of brook trout there are two sympatric populations: resident and sea run. The migratory behaviour of sea-run brook trout is likely to have been an ancestral behaviour that eventually was lost in some brook trout populations resulting in the current resident populations (Curry et al. 2010). These two distinct behaviours are important to preserve in river systems to ensure their biodiversity. Sea run populations of brook trout are declining, and their adaptation to utilize both marine and freshwater habitats could become a necessary adaption in changing river systems that would result in the preservation of the species. Using sulphur stable isotope analysis can help researchers determine if a brook trout sampled in the freshwater portion of a system is a resident or sea-run brook trout. In combination with other biological data, we can gain a better understanding of the differences between these two populations. Using sulphur in conjunction with carbon and nitrogen stable isotope analysis would provide a third isotope tracer. This could prove crucial to identifying food sources, especially for sea-run brook trout as they are likely feeding in both the marine and freshwater systems and therefore are feeding on several food sources. The methods for sulphur stable isotope analysis have been refined and are comparable to the methods for carbon and nitrogen analysis, so much so that it is possible to couple sulphur analysis with carbon and nitrogen analysis (Fry 2007).

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