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The effects of sex, tissue type, and dietary components on stable isotope discrimination factors ($\Delta^{13}C$ and $\Delta^{15}N$) in mammalian omnivores†

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We tested the effects of sex, tissue, and diet on stable isotope discrimination factors ($\Delta^{13}C$ and $\Delta^{15}N$) for six tissues from rats fed four diets with varied C and N sources, but comparable protein quality and quantity. The $\Delta^{13}C$ and $\Delta^{15}N$ values ranged from 1.7–4.1‰ and 0.4–4.3‰, respectively. Females had higher $\Delta^{15}N$ values than males because males grew larger, whereas $\Delta^{13}C$ values did not differ between sexes. Differences in $\Delta^{13}C$ values among tissue types increased with increasing variability in dietary carbon sources. The $\Delta^{15}N$ values increased with increasing dietary $\delta^{15}N$ values for all tissues except liver and serum, which have fast stable isotope turnover times, and differences in $\Delta^{15}N$ values among tissue types decreased with increasing dietary animal protein. Our results demonstrate that variability in dietary sources can affect $\Delta^{13}C$ values, protein source affects $\Delta^{15}N$ values even when protein quality and quantity are controlled, and the isotope turnover rate of a tissue can influence the degree to which diet affects $\Delta^{15}N$ values.

Keywords: animals; carbon-13; diet; food; isotope ecology; natural isotope fractionation; nitrogen-15; protein turnover; rats; $\delta^{13}C$; $\delta^{15}N$

1. Introduction

Natural variations in stable isotope ratios of carbon ($^{13}C/^{12}C$, reported as $\delta^{13}C$ values) and nitrogen ($^{15}N/^{14}N$, reported as $\delta^{15}N$ values) from animal tissues provide information on predator–prey relationships and can be used to make inferences about an individual's foraging ecology and habitat use [1]. Stable isotope ratios vary between animal tissues and their diets because of isotope fractionation and macronutrient routing, and this difference is called the trophic discrimination factor [2]. It is reported as $\Delta^{13}C$ for C and $\Delta^{15}N$ for N and is generally defined as $\Delta^{15}N_{\text{tissue-diet}} = \delta^{15}N_{\text{tissue}} - \delta^{15}N_{\text{diet}}$ (and similarly for C; see Methods).

One difficulty in the application of stable isotope analysis to modelling wild animal diets is the reliance on discrimination factors that are often assumed and not experimentally derived, as these values can vary depending upon many factors, including taxon, type of tissue analysed, growth rate, differences in digestive physiology, nutritional or reproductive status, the form of waste (i.e. excreta and nitrogenous waste), the macromolecular composition of diet (i.e. proportions of

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protein, carbohydrate, and lipid), and the extent of routing of dietary macromolecules to tissues composed of the same or different macromolecules (i.e. dietary protein to body protein, or dietary fat to body fat) [2–10]. Using assumed discrimination factors in stable isotope mixing models can result in errors in the estimation of wild animal foraging ecology [2,11,12]. For these reasons, it is important to estimate discrimination factors from captive animals and try to understand the ways in which the various factors listed above affect isotopic fractionation.

Several recent studies on captive animals have suggested that there is a linear relationship between the stable nitrogen isotope values of diet and trophic discrimination factors [13–16], and that the equations for these relationships can be used to estimate discrimination factors from dietary stable isotope values. There are no known physical or biochemical processes that could lead to different trophic fractionations based solely on the miniscule absolute differences in isotope concentration between different types of food [6,17]. Such correlations likely indicate experimental problems, such as animals that were not fully equilibrated to experimental diets before measurements were taken [17,18], or result from mathematical and statistical errors [18] and/or covariation between dietary isotope composition and some factor(s) that can affect trophic discrimination.

For example, $\Delta^{15}N$ values have been shown to increase with increasing dietary protein quantity [2,4,19,20], which is simply the amount of protein ingested. This may occur because animals eating higher quantities of protein may increase their waste flux to shed excess ingested nitrogen (generated by deamination of amino acids). As a greater fraction of body N excretion is ^{14}N-enriched waste, the body pool becomes ^{15}N-enriched [21–23]. In addition, $\Delta^{15}N$ values have been shown to decrease with increasing protein quality [6], which is the degree to which the amino acid composition of the diet matches the amino acid requirements of the consumer. If dietary amino acids are supplied in roughly the same relative abundance as in consumer tissues, and are supplied at a rate that meets daily needs, then the diet is considered to be of high protein quality. The $\Delta^{15}N$ values may decrease because, with increasing protein quality, the reproduction and excretion of ^{14}N-enriched waste are reduced [24]. It has proved difficult to measure the effects of dietary protein quantity and quality on diet–tissue $\Delta^{15}N$ values because these factors are difficult to separate [2]. Information on the effects of diet on animal $\Delta^{13}C$ values is lacking, but previous research suggests that diet type, including ingestion of C_3 vs. C_4 plant material, can effect carbon stable isotope discrimination factors [25,26].

We constructed a controlled feeding study using laboratory rats (*Rattus norvegicus*) to better understand how factors such as sex, tissue type, and dietary components influence the $\Delta^{15}N$ and $\Delta^{13}C$ values in mammalian omnivores, independent of dietary protein quantity and quality. We measured the $\Delta^{13}C$ and $\Delta^{15}N$ values from six tissues from rats held from birth for 276–278 days on four distinct diets. The diets were of nearly identical protein quantity (23.5–25.2 %; mean of 24.5 %) and quality as measured by amino acid content, but they differed in the source of their protein and carbon components, ranging from all plant material to two mixes of plant and animal material to animal only. We tested the hypotheses that there would be a) differences in the $\Delta^{13}C$ and $\Delta^{15}N$ values from rats depending upon the tissue type analysed, b) differences in the $\Delta^{13}C$ values depending upon dietary carbon sources, c) no differences in the $\Delta^{13}C$ and $\Delta^{15}N$ values within the same tissue types from rats due to sex, and d) no differences in the $\Delta^{15}N$ values within the same tissue types from rats regardless of diet type because the dietary protein quality and quantity were held relatively constant.

2. Methods

Eight sperm-positive Sprague–Dawley rats were received at the University of California Santa Cruz vivarium two days post-impregnation on 10 November 2005 from Charles River

Laboratories. Two each were randomly placed on one of four experimental diets (Table 1), each containing the same cellulose (fibre), vitamin, and mineral components, and nearly identical amino acid profiles – due to partial supplementation of three amino acids: methionine (all four diets), threonine (one diet), and lysine (two diets); (Tables 1 and 2) and proportions of calories from protein (23.5–25.2 %), carbohydrates (45.9–53.4 %), and fat (8.1–8.8 %) (Table 1), but protein and carbon components derived from different natural ingredients (Table 1). The amino acid levels of all diets exceeded those needed by rats for maintenance and growth (Table S1, see Online Supplemental Material), and so were considered to be of high protein quality.

To better investigate the role of different dietary components in determining trophic discrimination factors, the diets were designed to include isotopically distinct ingredients that encompassed a range of diet types that could be encountered by an omnivore. The protein components ranged from all-wheat gluten, a mix of wheat gluten and fish, a mix of wheat gluten, fish, chicken egg, and cow casein, and fish alone (Table 1). The carbohydrate and lipid components were derived from either C_3 (beet sugar and cottonseed oil, respectively) or C_4 (cane sugar and corn oil, respectively) plants, which allowed for a broad range of dietary carbon sources for examination (Table 1). All diets were manufactured by Harlan Teklad, USA, and individual ingredients were well mixed before incorporation into pellets to ensure uniform isotope compositions. Harlan Teklad specified the caloric and amino acid contents of all diets.

All females gave birth over a 24-hour period beginning on 8 December 2005 and all pups nursed from their mothers until weaning on 3 January 2006. After weaning, 6 pups (3 females and 3 males) were randomly chosen from each diet group (24 rats total) and held until 6–8 October 2006 (302–304 days from birth; 276–278 days total on the diet) on the diet their mothers consumed. Males and females were housed in separate cages and food and water were provided *ad libitum*. Rats maintained body mass and grew in an undetermined fashion throughout the experiment and were well passed full-grown by the end of the experiment. Rats were then euthanised via exposure to CO_2 (for approval see Acknowledgement), and sub-samples of blood, liver, kidney, muscle, and fur were removed, and blood was separated into cellular red blood cells (RBCs) and serum components. Blood was taken by syringe from the heart, muscle was taken from the upper right fore-leg, and fur was taken from the belly.

Tissues were cleaned with water (except blood components), freeze-dried for 24 hours, and lipids were removed from the rat tissues and all diet items (except the individual oil samples) using petroleum ether [27,28] in a Dionex ASE-200 Accelerated Solvent Extractor. Petroleum ether does not remove proteinaceous material and therefore does not affect δ^{15}N values [27,29]. Samples were ground to a powder using a mortar and pestle, 0.7–1.0 mg were sealed into tin capsules, and the δ^{13}C and δ^{15}N values were determined using a Carlo Erba CE1108 elemental analyser interfaced via a CONFLO III device to a Thermo-Electron Delta Plus XP mass spectrometer at the Stable Isotope Laboratory, University of California Santa Cruz. Precision for these data was determined using the standard deviations around the means for a subset of 47 internal laboratory standards (PU Gelatin) run at set intervals throughout analysis and was 0.15 ‰ for δ^{15}N and 0.05 ‰ for δ^{13}C.

The natural abundance of ^{13}C or ^{15}N in a sample is measured in relation to a standard and expressed in delta notation in this form:

$$\delta X = \frac{R_{\text{sample}} - R_{\text{standard}}}{R_{\text{standard}}} \times 1000, \tag{1}$$

where δX is the parts-per-thousand ('per mil') difference in isotopic composition between the sample and the standard, and R_{sample} and R_{standard} are the heavy-to-light isotope ratios (^{15}N/^{14}N and ^{13}C/^{12}C) of the sample and standard, respectively.

Table 1. The composition and macronutrient components of the four diets fed to rats in this study. Standard vitamin, mineral, and cellulose mixtures were added to all diets equally.

	Composition		Diet components and their isotope values (‰)							Whole diet isotope values (‰) and N and C concentration (±SD, $n = 3$)					
Diet	Protein source	Energy source[a]	Protein (% by weight)	Protein δ^{15}N	Protein δ^{13}C	Mean protein δ^{13}C[b]	Sucrose (% by weight)	Sucrose δ^{13}C	Oil (% by weight)	Oil δ^{13}C	δ^{15}N	δ^{13}C	% N	% C	C:N	Adjusted δ^{13}C
Wheat[c]	Wheat gluten[d]	C_3	23.5	4.7	−25.8	−25.8	Beet (53.4)	−24.9	Cottonseed (8.2)	−28.5	4.8 ± 0.1	−25.3 ± 0.0	4.4 ± 0.2	44.1 ± 0.4	10.1 ± 0.5	−25.6
Fish[e]	Fish meal[f]	C_4	25.2	12.5	−19.8	−19.8	Cane (45.9)	−11.7	Corn (8.8)	−14.9	12.3 ± 0.1	−16.2 ± 0.2	5.4 ± 0.3	43.3 ± 0.3	8.1 ± 0.6	−17.8
Wheat/fish[g]	Wheat gluten, fish meal	C_3	Total (24.3)			−23.8	Beet (51.8)	−25.0	Cottonseed (8.4)	−28.4	7.1 ± 0.0	−24.5 ± 0.0	5.1 ± 0.1	44.4 ± 0.3	8.8 ± 0.2	−25.2
			Wheat (15.7)	4.8	−25.9											
			Fish (8.6)	12.4	−19.9											
Wheat/fish/ casein/egg[h]	Wheat gluten, fish meal, cow casein[i], chicken egg whites[i]	C_4	Total (24.6)			−22.1	Cane (52.3)	−11.7	Corn (8.1)	−14.9	7.4 ± 0.1	−16.7 ± 0.2	4.6 ± 0.2	44.8 ± 0.4	9.7 ± 0.2	−19.5
			Wheat (9.1)	4.8	−25.9											
			Fish (8.8)	12.4	−19.9											
			Casein (4.6)	6.4	−27.1											
			Egg white (2.1)	4.3	−16.5											

Notes: Different amino acids were added to each diet to correct for potential deficiencies (see superscripts). Adjusted bulk diet δ^{13}C values were calculated using Equation (2) in the Methods (see supplemental materials for further discussion).

[a]Energy comprises sucrose and oil.

[b]The weighted average δ^{13}C value for all protein components together.

[c]The plant diet contained added lysine (7 g/kg), methioninie (3 g/kg), and threonine (1 g/kg).

[d]Wheat is a C_3 plant.

[e]The fish diet contained added methionine (2 g/kg).

[f]Fish meal comprises walleye pollock (*Theragra chalcogramma*) from the Bering Sea and produced by UniSea, Inc. (Dutch Harbor, AK).

[g]The wheat/fish diet had added lysine (2 g/kg) and methionine (2 g/kg).

[h]The wheat/fish/casein/egg diet had added methionine (2 g/kg).

[i]The cows and chickens were fed C_3- and C_4-based diets, respectively, and so their products are expected to exhibit these influences.

Table 2. Nitrogen and carbon trophic discrimination factors ($\Delta^{15}N$ and $\Delta^{13}C$, reported in ‰ ± SD) between diet (adjusted diet for C; see methods) and tissues for rats held on four experimental diets (see Table 1 for diet details).

	Female		Male		Female		Male	
Tissue	$\Delta^{15}N$	$\Delta^{13}C$	$\Delta^{15}N$	$\Delta^{13}C$	$\Delta^{15}N$	$\Delta^{13}C$	$\Delta^{15}N$	$\Delta^{13}C$
	Wheat diet				Fish diet			
Liver	+3.2 ± 0.1	+1.3 ± 0.3	+3.0 ± 0.2	+1.1 ± 0.8	+3.4 ± 0.2*	+0.9 ± 0.1	+2.8 ± 0.2*	+0.4 ± 0.4
Serum	+3.7 ± 0.1*	+1.6 ± 0.1	+3.5 ± 0.1*	+1.4 ± 0.0	+3.9 ± 0.1*	+0.6 ± 0.2	+3.4 ± 0.3*	+0.8 ± 0.2
Kidney	+2.8 ± 0.1*	+1.3 ± 0.1	+2.2 ± 0.2*	+1.4 ± 0.1	+3.4 ± 0.3*	+0.8 ± 0.2	+2.7 ± 0.1*	+0.8 ± 0.1
RBC	+2.2 ± 0.2*	+1.3 ± 0.1	+1.7 ± 0.2*	+1.2 ± 0.1	+3.2 ± 0.1*	+0.7 ± 0.1	+2.7 ± 0.1*	+0.6 ± 0.1
Muscle	+2.5 ± 0.1*	+1.7 ± 0.1	+2.0 ± 0.1*	+1.7 ± 0.2	+3.4 ± 0.2*	+1.2 ± 0.1	+2.7 ± 0.1*	+1.2 ± 0.1
Fur	+2.5 ± 0.1	+3.5 ± 0.2	+2.3 ± 0.2	+3.2 ± 0.1	+4.1 ± 0.2	+2.2 ± 0.1	+3.7 ± 0.2	+2.0 ± 0.2
	Wheat/fish diet				Wheat/fish/casein/egg white diet			
Liver	+3.4 ± 0.1*	+1.8 ± 0.5	+3.0 ± 0.1*	+1.1 ± 1.1	+3.2 ± 0.1*	+2.1 ± 0.0	+2.7 ± 0.1*	+2.2 ± 0.1
Serum	+3.9 ± 0.1*	+2.6 ± 0.1	+3.6 ± 0.1*	+2.6 ± 0.2	+3.9 ± 0.1*	+1.1 ± 0.0	+3.2 ± 0.0*	+1.2 ± 0.1
Kidney	+3.0 ± 0.3*	+2.6 ± 0.2	+2.3 ± 0.1*	+2.2 ± 0.3	+2.5 ± 0.2*	+1.4 ± 0.1	+1.9 ± 0.1*	+1.2 ± 0.1
RBC	+2.6 ± 0.1*	+2.7 ± 0.1	+2.2 ± 0.1*	+2.3 ± 0.3	+2.4 ± 0.2	+0.6 ± 0.1	+2.0 ± 0.1	+0.5 ± 0.1
Muscle	+3.0 ± 0.2*	+3.0 ± 0.1	+2.3 ± 0.1*	+2.5 ± 0.4	+2.5 ± 0.4	+1.4 ± 0.0	+2.1 ± 0.2	+1.5 ± 0.1
Fur	+2.9 ± 0.1	+4.3 ± 0.1*	+2.7 ± 0.1	+3.8 ± 0.2*	+3.0 ± 0.2	+2.3 ± 0.1*	+2.8 ± 0.1	+2.2 ± 0.1*

Notes: Tissues are listed in order of isotope turnover time from fastest (liver) to slowest (fur) [50]. Significant differences in $\Delta^{13}C$ and $\Delta^{15}N$ values between sexes held on the same diets are denoted by an asterisk (*). $N = 3$ females and 3 males per diet. The $\Delta^{13}C$ values between the $\delta^{13}C_{\text{bulk diet}}$ and $\delta^{13}C_{\text{rat tissues}}$ are reported in Table S2 and the mean $\Delta^{15}N$ and $\Delta^{13}C$ values for all rats together (females and males) for each diet are reported in Table S6 (see Online Supplemental Material).

Discrimination factors for N were determined by subtracting the mean values of the whole diet pellet from the mean $\delta^{15}N$ values of each tissue and are represented by $\Delta^{15}N$:

$$\delta^{15}N_{\text{tissue}} - \delta^{15}N_{\text{bulk diet}} = \Delta^{15}N. \quad (2)$$

Carbon in tissue protein can have multiple sources (dietary protein, carbohydrate, or lipid). Dietary protein is always preferentially routed to tissue protein; the minimum level is set by the essential amino acids in animal protein, which must come from the same amino acids in the diet. Earlier studies have shown that the extent of protein-to-protein routing depends on the macromolecular composition of the diet [30–33]. For rodents on a relatively high protein diet (a mean of 24.5 % for our study), C is derived primarily from dietary protein with a lesser amount coming from dietary carbohydrate and lipids [30,31,33,34]. Therefore, following recommendations in [30,35] and using values determined for rodents in [31], we accounted for routing and determined the discrimination factors for C using the equations:

$$(\delta^{13}C_{\text{protein}})(0.75) + (\delta^{13}C_{\text{carbohydrates}})(0.25) = \delta^{13}C_{\text{adjusted bulk diet}}, \quad (3)$$

$$\delta^{13}C_{\text{tissue}} - \delta^{13}C_{\text{adjusted bulk diet}} = \Delta^{13}C. \quad (4)$$

When more than one protein source contributed to the diet, the differential contributions of the various protein sources were accounted for, and we assumed that all protein sources were assimilated equally as indicated by protein digestibility studies [36,37]. As demonstrated by our results and the discussion below, this common assumption may be problematic. As with [31] we assumed that lipid was a minor component in new tissue production and, as MacAvoy et al. [31] only accounted for differential routing of carbon from the dietary protein and carbohydrate fractions of the diet into tissues for rats on high protein diets, we followed their example and did the same. However, we provide the calculations demonstrating that the routing of carbon from lipids would result in a 0.2 ‰ decrease in all $\Delta^{13}C$ values, which, as predicted in [31], is a very small contribution (see Supplemental Material for full description of these calculations). Most studies ignore effects of routing when calculating their $\Delta^{13}C$ values [24,38,39, i.e.]; for this reason, we have included the $\Delta^{13}C$ values determined from bulk diet $\delta^{13}C$ values in the Supplemental Materials (Table S2).

2.1. *Statistical analysis*

We used two-factor analyses of variance (ANOVA) to test for effects of tissue and diet type on the Δ^{13}C and Δ^{15}N values from female and male rats and to determine if there was an interaction between diet and tissue type. We then used single-factor ANOVAs to test for simple main effects of each diet type on the rats' Δ^{13}C and Δ^{15}N values for each tissue and of each tissue type on the rats' Δ^{13}C and Δ^{15}N values for each diet. These were followed by Tukey's pairwise comparisons to determine significant differences in discrimination factors among the different diet types and the different tissue types. We used *t*-tests to determine significant differences in the Δ^{13}C and Δ^{15}N values between sexes. Significance was tested at the $\alpha = 0.05$ level. Sample sizes were kept small ($n = 3$ female and 3 males rats held on each diet type) to avoid excess costs of housing, feeding, and sacrificing more animals than necessary. Despite the small samples sizes, we are confident in our results below as the variations around the mean stable isotope values as measured by their standard deviations were very small, ranging from 0.0 to 0.4 ‰ in most cases, and these small variations are close to biologically insignificant.

3. Results

3.1. *Variation in the $\Delta^{13}C$ and $\Delta^{15}N$ values*

Sex, tissue type, and diet type all contributed to the differences observed in the discrimination factors from rats. The ranges in Δ^{13}C and Δ^{15}N values were +0.4 to +4.3 ‰ and +1.7 to +4.1 ‰, respectively (Table 2; Figure 1; all δ^{13}C and δ^{15}N values are listed in Supplemental Table S3).

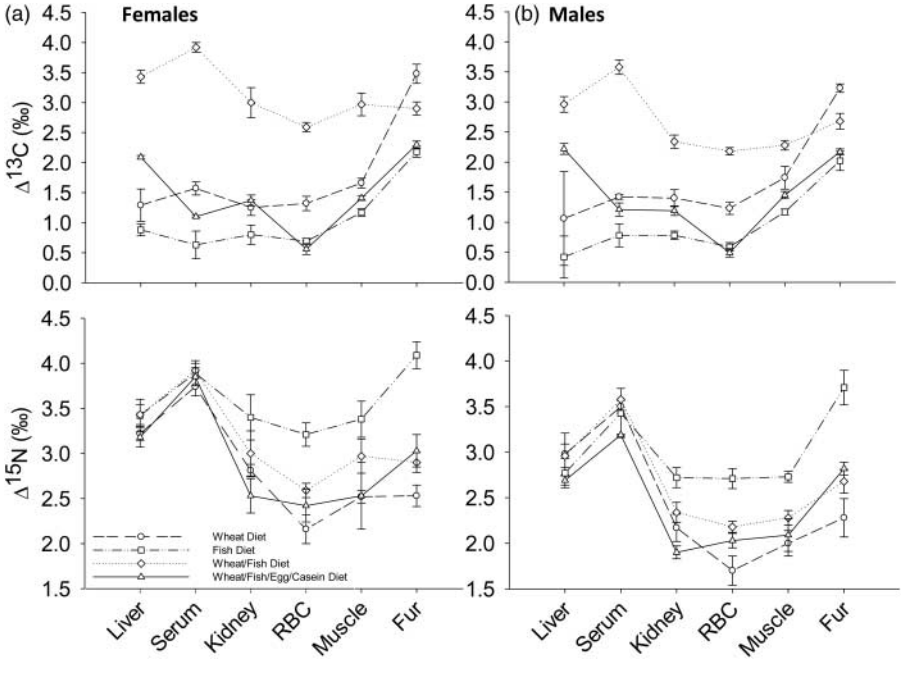

Figure 1. The variation in Δ^{13}C and Δ^{15}N values among tissue types from (a) female and (b) male rats held on the four experimental diets. Tissues are arranged along the *x* axis in order from fastest (liver) to slowest (fur) stable isotope turnover time [50]. $N = 3$ females and 3 males per diet.

The $\Delta^{13}C$ values were not different between sexes except in two cases where the $\Delta^{13}C$ values for fur from females were higher than those for males for rats held on the wheat/fish and the wheat/fish/casein/egg diets (Table 2, Table S3). In contrast, most of the $\Delta^{15}N$ values from all tissues on all diets were significantly higher for females by 0.2–0.7 ‰ except in a few cases where the $\Delta^{15}N$ values were not different between sexes. The $\Delta^{15}N$ values were the same between sexes for fur from rats on all diets, liver from rats on the wheat diet, and muscle and RBCs from rats held on the wheat/fish/casein/egg diet (Table 2, Table S3).

Two-factor ANOVAs designed to test for the effects of tissue type and diet type on the isotope discrimination factors and to test for an interaction effect between the two main effects demonstrated significant interactions between tissue and diet types for the $\Delta^{13}C$ (females: $F_{15,48} = 19.2$, $p < 0.01$; males: $F_{15,48} = 6.1$, $p < 0.01$) and $\Delta^{15}N$ (females: $F_{15,48} = 7.3$, $p < 0.01$; males: $F_{15,48} = 12.3$, $p < 0.01$) values (Figure 1). Therefore, the effects of tissue type on the $\Delta^{13}C$ and $\Delta^{15}N$ values were not independent of diet type (and vice versa). Using the sum of squares data from the two-factor ANOVA results, we determined that the interaction effect accounted for the smallest amount of variability (10.7 to 16.8 % of the total sum of squares, depending upon sex and $\Delta^{13}C$ or $\Delta^{15}N$ values); tissue type accounted for most of the variability (53.3 % for females, 63.7 % for males, vs. 26.9 and 17.0 %, respectively, for diet type) observed in the $\Delta^{15}N$ values, whereas tissue (39.2 % for females, 39.6 % for males) and diet (48.3 % for females, 34.7 % for males) types accounted for similar amounts of variability observed in the $\Delta^{13}C$ values.

To further examine the impacts of the main effects on the discrimination factors, we used single-factor ANOVAs and post-hoc Tukey's Honestly Significant tests to test tissue and diet types separately. There were significant differences in the $\Delta^{13}C$ and $\Delta^{15}N$ values among tissue types for rats held on the same diets (Table S4) and among diet types across the same rat tissues (Table S5).

3.2. *Effects of tissue type on the $\Delta^{13}C$ and $\Delta^{15}N$ values from rats held on each of four diets*

We examined the $\Delta^{13}C$ and $\Delta^{15}N$ values from rats for each diet type to see how they varied depending upon the tissue type (Figure 1, Table S4). Except for rats on the wheat/fish diet, fur generally had significantly higher $\Delta^{13}C$ values than all other tissues, but the tissues with the lowest $\Delta^{13}C$ values varied depending upon the diet (Table 2, Figure 1, Table S5). The differences in the $\Delta^{13}C$ values among tissues for all rats increased with the addition of more varied carbon sources into the diet (Table 2, Figure 1, Table S4). For example, the $\Delta^{13}C$ values from all tissues from male rats held on the C_3 plant-based wheat diet were equal except for those from fur, which were significantly higher than for all other tissues. Females demonstrated the same pattern except the $\Delta^{13}C$ values from muscle were also different than those from liver. In contrast, the $\Delta^{13}C$ values from nearly all tissues were different from one another for rats on the mixed C_3 and C_4 plant-based wheat/fish/casein/egg diet. Differences in the $\Delta^{13}C$ values from rats on the fish and the wheat/fish diets were intermediate between these two extremes.

Differences in the $\Delta^{15}N$ values among tissues also differed greatly depending upon the diet source, and there were more differences in $\Delta^{15}N$ values among tissues held on a particular diet than there were for $\Delta^{13}C$ values among tissues. The number of significant differences (out of 15 comparisons) in the $\Delta^{15}N$ values among tissues was highest for the rats held on the wheat diet (11–14), nearly the same for the rats held on the two mixed diets (9–11), and lowest for the rats held on the fish diet (8–9) (Figure 1, Table S4).

In general, the $\Delta^{15}N$ values from serum and liver were always greater than the $\Delta^{15}N$ values from all other tissues, regardless of diet, except for rats held on the fish diet. The $\Delta^{15}N$ values for serum were the highest across all diets except when the $\Delta^{15}N$ values from serum were equal to those from fur for all rats on the fish diet. Liver had the second highest $\Delta^{15}N$ values except when

they were equal to fur on the wheat/fish/casein/egg diet and equal to RBCs, muscle, and kidney on the fish diet. RBCs generally had the lowest Δ^{15}N values (Table 2, Figure 1, Table S4).

3.3. *Effects of diet type on the $\Delta^{13}C$ and $\Delta^{15}N$ values for each of the six tissue types*

We examined the Δ^{13}C and Δ^{15}N values from rats for each tissue type to see how they varied depending upon the diet type (Table 2, Figure 1, Table S5). All serum from all rats had the same Δ^{15}N values regardless of diet type and the same was true for liver tissues (Table 2, Figure 1, Table S5). We observed a similar pattern for the Δ^{13}C values for liver tissues from males, which showed only one difference in the Δ^{13}C values between the fish and wheat/fish/casein/egg diets. The Δ^{13}C values for liver tissues from females were different in three of the six comparisons (wheat/fish/casein/egg vs. wheat diets, wheat/fish/casein/egg vs. fish diets, and wheat/fish vs. fish diets). We did not observe this pattern for the Δ^{13}C values from serum, which were all different among diet types (except for those held on the wheat and wheat/fish/casein/eggs diets) (Table 2, Figure 1, Table S5).

We also found that in all but one case (kidney tissue from males) the Δ^{15}N values were the same for a specific tissue if the rats were held on one of the two diets that contained mixed animal and vegetable protein (wheat/fish and wheat/fish/casein/egg). The exact opposite pattern was observed for Δ^{13}C values; they were all different from one another between the two mixed diet types, except for liver tissues, which were the same. Finally, there were positive linear relationships between the dietary δ^{15}N values, which are proxies for the trophic levels of each diet, and the Δ^{15}N values for fur, muscle, RBCs, and kidney tissues, but not liver or serum (Figure 2).

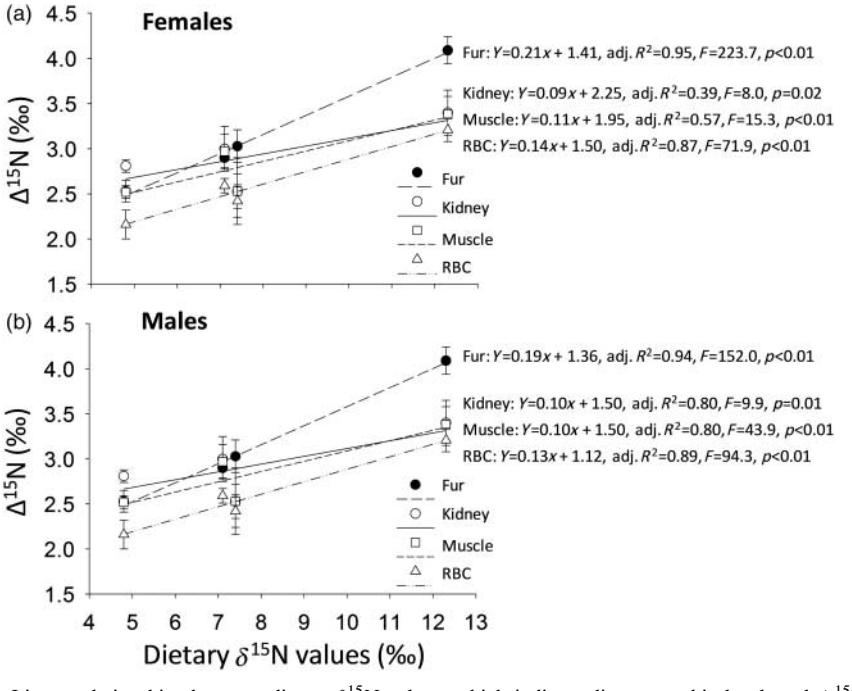

Figure 2. Linear relationships between dietary δ^{15}N values, which indicate dietary trophic level, and Δ^{15}N values (‰; ±SD) for rats for four of the six measured tissues. All dietary data are in Table 1. There were no linear relationships between dietary δ^{15}N values and the Δ^{15}N values for liver and serum as the Δ^{15}N values were equal among all diets for these tissues.

4. Discussion

There were almost no differences in the $\Delta^{13}C$ values between sexes, whereas, in most cases, females had higher $\Delta^{15}N$ values than males. This is likely a result of differential amounts of growth between sexes over the course of the study. Growing animals are known to have reduced $\Delta^{15}N$ values [2,40] because they retain more ^{14}N in their body pool as they route more dietary protein to tissue deposition rather than removing N as waste. Consequently, the pool of N remaining in the body to synthesise tissues in growing animals contains more ^{14}N, which leads to lower $\delta^{15}N$ (and thus $\Delta^{15}N$) values than those observed for animals at stasis [2,40]. All rats were considered mature adults well in advance of the end of the experiment (they were 302 to 304 days old on the final day of the experiment), but males grew longer and heavier as demonstrated by the final mean weights and lengths from females and males which were (471.8 ± 72.2 SD) g vs. (827.5 ± 116 SD) g, respectively, and (24.0 ± 0.7 SD) cm vs. (28.6 ± 0.9 SD) cm, respectively (*t*-tests; weight: $p < 0.01$, $t = -9.0$, df = 22; length: $p < 0.01$, $t = -13.2$, df = 22). The added growth in terms of extra mass and length observed in the males likely resulted in lower $\delta^{15}N$ and thus lower $\Delta^{15}N$ values.

Studies of wild animal trophic ecology frequently attribute differences in stable isotope values between sexes to variations in diet or foraging location [i.e. 41,42]. Our data suggest that sex differences in stable isotope values may be due to attributes of growth not related to diet and may need to be considered when analysing wild animal isotope data. This could be especially important in species where sexual dimorphism is pronounced or when other factors such as pregnancy and lactation influence tissue deposition and loss rates from females [40].

The two-factor ANOVA results indicated that tissue type explained the greatest amount of variation in the $\Delta^{15}N$ values (53–63 %), whereas tissue and diet contributed to the variation observed in the $\Delta^{13}C$ values to a similar degree (~40 %). Our work confirms previous studies demonstrating differences in stable isotope discrimination factors among tissue types for mammals held on constant diets [2,30,39,43,44]. These differences are likely due to the distinct amino acid composition of each tissue because amino acids have differing $\delta^{13}C$ and $\delta^{15}N$ values [2,45–47].

The range in $\Delta^{15}N$ values observed among tissues from rats held on the same diet in this study was not large, from 0.8 ‰ for female rats held on the wheat/fish/casein/egg diet to 1.8 ‰ for male rats held on the wheat diet. Despite this small range of values, in some cases, this difference could represent one entire trophic level (as seen with some of the $\Delta^{15}N$ values in our study which are around 1.7 to 2.0 ‰) and so merits attention when applying $\Delta^{15}N$ values for a particular tissue type to the interpretation of wild animal foraging ecology data.

The ranges in $\Delta^{13}C$ values among tissues for rats held on the same diet ranged from 1.6 ‰ for all rats on the fish diet to 2.7 ‰ for male rats on the wheat/fish diet, further underscoring the need to apply the best discrimination factor when estimating diets based on stable isotope values from particular tissues using isotope mixing models. In addition, the $\Delta^{13}C$ values from fur were considerably higher than all or most of the other more metabolically active tissues, regardless of diet type. This is a pattern frequently observed in the $\Delta^{13}C$ values from keratinous tissues [7,26,38,43,48–52] and is largely attributed to the unique amino acid composition of α-keratin, the structural material composing fur and whiskers [53,54]. Glycine, serine, and glutamate are the primary components of α-keratin [54] and they have higher $\delta^{13}C$ values relative to other amino acids [55,56] which would contribute to the high $\Delta^{13}C$ values observed for keratinous tissues. As fur is frequently a targeted tissue for stable isotope analysis of wild animals due to the non-invasive nature of its collection, care should be taken to consider its potential to exhibit higher $\Delta^{13}C$ values when using it to estimate wild animal foraging ecology.

Differences in amino acid composition among tissue types are only one factor contributing to the isotopic variation observed among tissues. Variations in the isotopic composition of dietary sources that contribute to an animal's bulk diet may also drive differences in $\Delta^{13}C$ and $\Delta^{15}N$

values among the different tissue types. For example, our data suggest that variations in the $\Delta^{13}C$ values among tissues increased when the $\delta^{13}C$ values of the dietary carbon sources were highly variable, whereas they were minimal when the dietary $\delta^{13}C$ values were constrained. The wheat diet provided such a constraint as wheat is a C_3 plant, and the sucrose and lipid components of the wheat diet were also derived from C_3 plants (beets and cottonseed oil, respectively). Therefore, all available carbon routed to all tissues came from C_3 plants with fairly uniform $\delta^{13}C$ values (-28.5 to -24.9 ‰), potentially obscuring any variations induced by routing that we might have failed to account for, and the $\Delta^{13}C$ values were all equal among tissues (except for fur; see above) for rats held on the wheat diet.

In contrast, the $\Delta^{13}C$ values among tissues were almost entirely different from one another for rats held on the more complex wheat/fish/casein/egg diet. This diet contained protein from wheat gluten (a C_3 plant), fish, and casein and egg products derived from animals that had consumed C_3 and C_4 plant material, respectively. It also contained energy sources from C_4 plant-derived sucrose and lipid (cane sugar and corn oil, respectively). Thus, it appears that the varying carbon sources, which ranged in their $\delta^{13}C$ values from -11.7 ‰ (cane sugar) to -27.1 ‰ (egg whites), increased the potential for variation in the $\delta^{13}C$ (and thus $\Delta^{13}C$) values among tissue types and explains the high variation among tissues we observed. This follows from another study suggesting that C_3 vs. C_4 plant-based diets can cause variations in isotope discrimination factors for animals [25].

If there were no isotopic routing and all carbon molecules were evenly distributed throughout the body, then variability in dietary macromolecule $\delta^{13}C$ values would not matter and there would be no differences in $\Delta^{13}C$ values among tissues regardless of diet. Our data demonstrate that isotopic routing occurs and the variability in dietary $\delta^{13}C$ values contributed to the variation observed in the $\Delta^{13}C$ values among tissues. Because wild omnivores likely consume diets containing components with highly variable $\delta^{13}C$ values, it is important to proceed cautiously when using $\Delta^{13}C$ values derived from captive omnivores held on diets that are overly simplified and do not mimic a wild omnivore diet.

Differences in the $\Delta^{15}N$ values among tissue types were minimised when dietary sources for nitrogen came entirely from a single animal source (fish diet) and they were maximised when the rat diet was composed entirely of plant material (wheat diet), indicating that diet type mattered for consistency of $\Delta^{15}N$ values. We also observed a positive correlation between dietary $\delta^{15}N$ values, which are a reflection of trophic level or the amount of dietary protein derived from plants vs. animals and are measured by the bulk diet $\delta^{15}N$ values, and the $\Delta^{15}N$ values from kidney, muscle, RBCs, and fur (Figure 2; linear regressions, all $p < 0.01$). Fur is the tissue with the slowest isotopic turnover time, and kidney, muscle, and RBCs exhibit intermediate isotope turnover times [50]. We did not observe this relationship between dietary trophic level and the $\Delta^{15}N$ values from liver and serum. These are the two tissues with the fastest isotope turnover times [50], and their $\Delta^{15}N$ values were the same, regardless of diet, demonstrating no relationships with dietary trophic level. Therefore, increases in dietary trophic level appear to cause elevated $\Delta^{15}N$ values in tissues from rats, but this pattern applies only to tissues with medium to slow stable isotope turnover times.

The positive relationship between dietary trophic level and $\Delta^{15}N$ values matches patterns observed in birds [4] and in a review of aquatic invertebrates and muscle from fish [34], and it has been demonstrated that animals at steady states can have varying $\Delta^{15}N$ ratios depending upon their dietary protein source [20]. For instance, carnivores on a high protein animal diet have higher $\Delta^{15}N$ values because they have to increase their waste flux to shed excess ingested protein; as a greater fraction of body N is excreted as ^{14}N-enriched waste, the body pool becomes ^{15}N-enriched [21–23]. Herbivores on a lower protein plant diet have lower $\Delta^{15}N$ values because they have a much lower waste flux and so retain more ^{14}N in tissues [22]. As the amounts of protein (23.5 % for the wheat diet to 25.2 % for the fish diet) and the quality of protein, as measured by dietary amino acid content and amino acid needs of the rats (Table S5), were fairly constant among diet types,

it appears that the actual components of the diet, plant vs. animal protein, drove the differences in the Δ^{15}N values observed among diet types. This could be caused by variable digestibility of the dietary ingredients as one study found the nitrogen digestibility of fish as a protein source for rats is 1.7 times higher than that for wheat gluten and 1.2 times higher than that for casein ((52 ± 3) g/16 g N vs. (30 ± 2) g/16 g N vs. (42 ± 4) g/16 g N, respectively; all error terms are SE) [57]. This contradicts other studies [36,37] that found very similar protein digestibility values for fish, wheat gluten, and casein in rats. If there is a digestibility difference between plant and animal material, then omnivores with more potentially highly digestible animal-derived protein in their diets may simply have greater amounts of available dietary protein leading to greater waste flux and retention of ^{15}N over those consuming more plant material that may be less digestible. Clearly, this issue needs further study.

The pattern of increasing Δ^{15}N value with dietary δ^{15}N values only applied to tissues with slow to medium isotope turnover times, and no such relationship was observed for the Δ^{15}N values from liver and serum. These two tissues have the fastest isotope incorporation rates or turnover times in our study [50], and they generally had the highest Δ^{15}N values across all diets except for fur from animals held on the fish diet. Tissues are constantly broken down and regenerated, and the amino acids in body fluids that are used to build proteins come from both degrading tissues (endogenous sources) and diet (exogenous sources). All amino acids are mixed in the blood, and the speed at which a tissue reaches equilibrium with a dietary amino acid source is correlated with the metabolic activity of that particular tissue and the overall metabolic rate of the animal in question [43,58,59].

It has been speculated that tissues with very high metabolic rates (and faster stable isotope turnover rates) would have larger Δ^{15}N values because nitrogen requirements increase with metabolic rate leading to higher levels of preferential excretion of ^{14}N in waste products [35,60]. In addition, high metabolic tissues undergo fast protein breakdown and replacement, resulting in frequent re-use of their proteins and further exposing their components to enzymatic fractionation causing greater accumulation of ^{15}N [8,30,31,35]. As serum and liver showed no differences in Δ^{15}N values between diet types, it appears that the effect of diet type is ameliorated by the rapid breakdown and re-use of the exogenous and endogenous proteins as described above. However, we cannot explain why this occurred and recommend further study to determine why the Δ^{15}N values from tissues with very fast metabolic rates do not appear to be affected by variations in dietary components.

Our pattern of increasing Δ^{15}N values with increasing dietary δ^{15}N value is opposite to the trend reported by Robbins et al. [6] and Poupin et al. [8] and their hypotheses which predict that animals foraging at higher trophic levels will have smaller diet–tissue Δ^{15}N values. It also contradicts patterns observed in a previous study of rats [13] and in a review of mammals, birds, fish, and invertebrates [16], both of which have since been shown to have mathematical, statistical, and methodological problems [17,18]. Our data support the conclusions put forth by Perga and Grey [17] and Auerswald et al. [18] and add to their analyses.

Part of the difficulties with the aforementioned studies is that the authors probably did not hold their animals on their experimental diets for a time long enough to measure true discrimination factors. The authors of [13] used adult rats that had been held elsewhere on an unspecified diet before starting them on their experimental diets, whereas the authors of [6] and [8] purchased adult rats, held them in their labs on rat chow for seven days, then switched them to their experimental diets. In our study, baby rats were obtained from mothers that were started on the experimental diets 2 days post-impregnation. These baby rats were born, nursed by their mothers for 26 days, switched to their experimental diets, and held on those diets for 275–277 days. The rats in [13], [6], and [8] were held on their experimental diets for 56 (liver, muscle, and fur), 21 (plasma), and 21 (small intestine mucosa, liver, plasma, kidney, stomach, and colon) days, respectively. It has been demonstrated that it takes approximately 25–50, 50–75, 75–100, 200, and 275 days,

respectively, for δ^{15}N values in serum (comparable to plasma), liver, kidney, muscle, and fur from rats to fully equilibrate isotopically to a new diet after a switch (see Supplemental Figures S1, S2, S3, S5, and S6 in [50]). Therefore, even the tissues with the most rapid turnover (liver and plasma) may not have fully equilibrated to the experimental diets in any of these studies. If so, the Δ^{15}N values were calculated by comparing experimental diet δ^{15}N values with the δ^{15}N values from rat tissues that still contained substantial fractions of amino acids incorporated from diets fed to the rats prior to their use in the experiments. The one possible exception is the stomach intestine mucosa from [8] which may have been equilibrated with their experimental diet and had Δ^{15}N values that were not different between soy or milk protein diet types.

The trend (decreasing Δ^{15}N values with increasing dietary δ^{15}N values) observed in [6,8,13] does fit with the following scenario using isotope analysis of fur as an example. A standard rodent diet used in laboratories is a mix of corn and wheat with a δ^{15}N value of ~2.8 ‰ [30]. Therefore, fur tissue from adult rats held on a diet of ~2.8 ‰, then switched to an experimental diet and held for 56 days, would only be ~20 % equilibrated between the new and old diets (i.e. 56-day experiment vs. at least 275 days for full equilibration) (see Supplemental Figure S6 in [50]). The δ^{15}N values for fur from these rats would be lower than expected if the experimental diets were greater than ~2.8 ‰ and higher than expected if the experimental diets were less than ~2.8 ‰. That is exactly the pattern observed for all rat tissues in the mentioned studies: lower Δ^{15}N values between rat tissues and diet for animals held on diets with higher δ^{15}N values (animal protein diets) and higher Δ^{15}N values for rats on diets with lower δ^{15}N values (plant protein diets) and is likely the reason for the patterns they observed.

One of the studies [13] also showed Δ^{13}C values for liver, muscle, and fur tissue from rats which were extremely wide-ranging and very different from those presented in this study. One explanation for this discrepancy is that they did not account for the fact that carbon incorporated into rodents on relatively high protein diets, such as was used in this and their study, derives primarily from protein [30,31,35] (see Methods). The clearest sign of this is the fact that they found negative Δ^{13}C values between all of the tissues and most of the experimental diets. In our study, the Δ^{13}C values calculated without accounting for C routing (see Table S2) were lower than those calculated using the δ^{13}C values from the adjusted bulk diet that did account for C routing (see Table 2). For rats held on the fish and the wheat/fish/casein/egg diets, these differences were large enough (1.6 and 2.8 ‰, respectively) to result in negative Δ^{13}C values. This makes little biological sense and could confound the discrimination factors determined in studies that do not take carbon routing into account. In addition, the Δ^{13}C values found in [13] suffered the same problems noted above regarding the insufficient amount of time for the equilibration. Finally, the authors of [13] did not lipid extract their diet items or rat tissues, leading to further complications, as lipids are significantly ^{13}C-depleted [3,61,62], which likely also contributed to the very low δ^{13}C values observed in their study.

5. Conclusions

We found that differential growth over the course of the study likely led to slightly higher Δ^{15}N values for females over males, whereas the Δ^{13}C values were not affected. The Δ^{13}C values differed more among tissue types as the δ^{13}C values of the dietary carbon sources increased in variability, whereas differences in the Δ^{15}N values among tissue types decreased with increasing dietary animal protein. The Δ^{15}N values increased with increasing dietary δ^{15}N value, which indicates dietary trophic level for all tissues except liver and serum, indicating that, even when dietary protein quality (as measured by dietary amino acid content) is held constant, variation in the trophic level of dietary sources can affect Δ^{15}N values. This

effect was not observed in liver and serum, tissues with very fast stable isotope turnover times, indicating that the stable isotope turnover time of a particular tissue can also influence $\Delta^{15}N$ values.

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