
Metabolic Routing of Dietary Nutrients in Birds: Effects of Diet Quality and Macronutrient Composition Revealed Using Stable Isotopes

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ABSTRACT

During fall migration many songbirds switch from consuming primarily insects to consuming mostly fruit. Fruits with more carbohydrates and less protein may be sufficient to rebuild expended fat stores, but such fruits may be inadequate to replace catabolized protein. We manipulated the concentrations and isotopic signatures of macronutrients in diets fed to birds to study the effects of diet quality on metabolic routing of dietary nutrients. We estimated that approximately 45% and 75%, respectively, of the carbon in proteinaceous tissue of birds switched to high- or low-protein diets came from nonprotein dietary sources. In contrast, we estimated that approximately 100% and 20%–80%, respectively, of the nitrogen in proteinaceous tissues of birds switched to high- or low-protein diets was attributable to dietary protein. Thus, the routing and assimilation of dietary carbon and nitrogen differed depending on diet composition. As a result, $\delta^{15}\text{N}$ of tissues collected from wild animals that consume high-quality diets may reliably indicate the dietary protein source, whereas $\delta^{13}\text{C}$ of these same tissues is likely the product of metabolic routing of carbon from several macronutrients. These results have implications for how isotopic discrimination is best estimated and how we can study macronutrient routing in wild animals.

Introduction

Many songbirds are omnivorous and switch their diets from insects to fruits during migration (Bairlein 1987, 1990). Fruits

and insects differ in nutritional value; fruits on average are lower in protein and higher in carbohydrates than insects (Levey and Karasov 1989; Bairlein 1996; Witmer 1998). At many stopover sites during migration, songbirds consume large amounts of fruit that may be sufficient to rebuild expended fat stores (Bairlein 1987; Izhaki and Safriel 1989; Parrish 1997, 2000). However, songbirds also catabolize protein during migration, and diets low in protein may not be sufficient to rebuild proteinaceous tissue (Bauchinger and Biebach 1998; Karasov and Pinshow 1998; Bordel and Haase 2000; Schwilch et al. 2002). Determining whether certain fruits or some mix of fruits and insects are adequate for replenishing fat and protein stores in migratory birds requires understanding how dietary nutrients are metabolically routed to bird tissues.

Stable isotope signatures in natural foods can be used to study metabolic routing of dietary nutrients to tissues in free-living birds, although few studies have quantified the influence of diet quality on rebuilding and replenishing fat and protein stores in songbirds (Gannes et al. 1997; Kelly 2000). Given that birds need to rebuild proteinaceous tissue at stopover sites and that dietary protein may be preferentially routed to proteinaceous tissues (Martínez del Río and Wolf 2005), the isotopic signature of proteinaceous tissues in birds should be similar to the isotopic signature of dietary protein for birds consuming nutritionally adequate diets such as insects. Recent research has shown that the isotopic signatures of carbon and nitrogen in proteinaceous tissue assimilate at similar rates, and thus the metabolic pathways of tissue formation are considered coupled for carbon and nitrogen (Hobson et al. 2000; Haramis et al. 2001; Evans-Ogden et al. 2004). However, if birds eat primarily fruit with less protein, there may be a decoupling of the carbon and nitrogen metabolic pathways of tissue formation (Hobson et al. 2000; Bearhop et al. 2002; Hobson and Bairlein 2003; Pearson et al. 2003). As a result, birds that consume low-protein fruits may route carbon from nonprotein sources into proteinaceous tissue.

The few studies that have used stable isotopes to investigate differences in metabolic routing for animals fed specific diets did not find differences in assimilation of carbon and nitrogen into proteinaceous tissues (Hilderbrand et al. 1996; Haramis et al. 2001; Bearhop et al. 2002; Evans-Ogden et al. 2004). However, much of the previous research on turnover and metabolic routing of carbon and nitrogen has been conducted on animals fed diets with relatively homogeneous isotopic signatures for

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the macronutrients (carbohydrate, lipid, and protein, all from the same source). If both dietary carbohydrate and protein supply carbon for protein synthesis, then detecting differences between carbon and nitrogen routing may not be possible using diets with homogeneous macronutrient signatures. Differences in carbon and nitrogen routing may become apparent in animals fed diets with heterogeneous macronutrient signatures because animals may route dietary carbon and nitrogen differently depending on diet composition (Bearhop et al. 2002; Pearson et al. 2003), and there may be differences in metabolic routing between tissues within the same individual (Hobson and Bairlein 2003).

Metabolic routing of dietary macronutrients could also affect discrimination between bulk diet and consumer tissue ($\Delta_{dt} = \delta_{\text{tissue}} - \delta_{\text{diet}}$). Ecologists commonly use discrimination values to construct trophic relationships (Hobson et al. 2000; Post 2002) and to reconstruct the contributions of multiple food components to an animal's diet (Ben-David et al. 1997; Paszkowski et al. 2004). However, discrimination values for proteinaceous tissues may be more accurately calculated between dietary protein and tissue than between bulk diet and tissue (Podlesak et al. 2005). As a consequence, estimates of trophic position and of the relative contributions of different components to an animal's diet using bulk-diet tissue discrimination values could be inaccurate if animals metabolically route dietary macronutrients differently because of differences in diet composition.

Our primary objective was to use stable isotopes of carbon and nitrogen to investigate macronutrient routing in yellow-rumped warblers (*Dendroica coronata*) fed diets with different macronutrient concentrations and isotopic signatures. We

tested the hypothesis that the carbon and nitrogen isotopic signature of proteinaceous tissues in birds fed nutritionally adequate diets is similar to that of dietary protein. We also tested the hypothesis that birds fed diets with less protein must metabolically route carbon from nonprotein dietary sources into protein synthesis.

Material and Methods

We captured yellow-rumped warblers during fall migration in 2001 and 2002, on Block Island, Rhode Island (41°12'N, 71°35'W; U.S. Fish and Wildlife Service permit 22923-A, Rhode Island Department of Environmental Management permit 2001-75C). Yellow-rumped warblers are highly omnivorous and consume large amounts of fruit at such stopover sites along the New England coast (Parrish 1997). Birds were transferred to the animal care facilities on the University of Rhode Island's Kingston campus and housed in stainless steel cages (51 cm × 35.5 cm × 20.5 cm) in a room with a constant light cycle (12L : 12D) and temperature (21°C). Sixty-nine birds captured during 2001 were randomly assigned to either a C₃-based diet ($n = 34$) or a C₄-based diet ($n = 35$) with the same macronutrient composition (25% protein, 53% carbohydrate, 12% fat) but formulated with different ingredients (Tables 1, 2; Fig. 1). As formulated, $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, and percent carbon were different between the two acclimation diets, but the percent nitrogen was not different (Table 2). Throughout the experiment, birds had ad lib. food and water, and each day they were provided five waxworms (*Galleria mellonella*; approximately 1 g wet; $\delta^{13}\text{C} = -20.8\text{‰}$; $\delta^{15}\text{N} = 7.0\text{‰}$) to ensure birds maintained body mass (Frazer and McWilliams 2002). To determine the

Table 1: Ingredients used in the acclimation and experimental diets of yellow-rumped warblers

Source and Macronutrient	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰) ^a	%C	%N ^a	n
C ₃ :					
Beet sugar	$-24.5 \pm .6$		$39.3 \pm .2$		3
Casein	$-26.0 \pm .7$	$5.9 \pm .8$	$46.5 \pm .2$	$14.4 \pm .2$	4
Olive oil	$-28.9 \pm .3$		73.3 ± 1.8		3
C ₄ :					
Corn sugar	$-9.8 \pm .1$		$37.8 \pm .9$		4
Fish meal ^b	$-18.4 \pm .1$	$14.1 \pm .1$	40.9 ± 2.4	$10.4 \pm .7$	3
Corn oil	$-15.3 \pm .6$		70.5 ± 1.1		3

Note. $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, percent carbon (%C), and percent nitrogen (%N; mean \pm 1 SD) of the ingredients in the C₃ and C₄ acclimation diets fed to yellow-rumped warblers during the 2.5-mo acclimation period and in the four experimental diets fed to yellow-rumped warblers during the 15-d feeding trial. n = number of different ingredient sources used during the experiment. Some birds were supplemented with five waxworms per day (waxworm: protein, $\delta^{13}\text{C} = -18.6\text{‰} \pm 0.1\text{‰}$, $\delta^{15}\text{N} = 7.0\text{‰}$; lipid, $\delta^{13}\text{C} = -22.7\text{‰} \pm 0.0\text{‰}$).

^a No measurable nitrogen in sugars or oils.

^b Fish meal is composed of approximately 70% protein ($\delta^{13}\text{C} = -17.0\text{‰} \pm 0.2\text{‰}$), 10% fat ($\delta^{13}\text{C} = -23.8\text{‰} \pm 0.0\text{‰}$), and 20% ash.

Table 2: Acclimation and experimental diets fed to yellow-rumped warblers

Diet	% Dry Mass			Macronutrient Source			Whole-Diet Signature		Whole-Diet Composition		<i>n</i>
	Protein	Carb	Lipid	Protein	Carb	Lipid	$\delta^{13}\text{C}$ (‰)	$\delta^{15}\text{N}$ (‰)	%C	%N	
C ₃	25	53	12	C ₃	C ₃	C ₃	-25.2 ± .2 ^A	5.5 ± .5 ^A	46.1 ± .5 ^A	3.7 ± .1 ^A	7
C ₄	25	53	12	C ₄	C ₄	C ₄	-13.7 ± .4 ^B	13.8 ± .9 ^B	41.4 ± 1.1 ^B	3.6 ± .1 ^A	7
1	25	53	12	C ₄	C ₃	C ₃	-22.8 ± .1 ^C	14.2 ± .2 ^B	43.3 ± 1.3 ^{BC}	3.7 ± .1 ^A	3
2	25	53	12	C ₃	C ₄	C ₄	-16.7 ± .3 ^D	5.5 ± .1 ^A	45.4 ± .3 ^{ACD}	3.5 ± .2 ^A	3
3	4	74	12	C ₄	C ₃	C ₃	-24.7 ± .1 ^A	14.3 ± .6 ^B	43.6 ± 1.8 ^{BD}	.6 ± .1 ^B	3
4	4	74	12	C ₃	C ₄	C ₄	-12.5 ± .0 ^E	5.5 ± .2 ^A	41.2 ± .2 ^B	.6 ± .1 ^B	3

Note. Composition (% dry mass), macronutrient source (C₃ or C₄), and whole-diet $\delta^{13}\text{C}$, $\delta^{15}\text{N}$, %C, and %N (mean ± 1 SD) of the C₃ and C₄ acclimation diets fed to yellow-rumped warblers during the 2.5-mo acclimation period and of the four experimental diets fed to yellow-rumped warblers during the 15-d feeding trial. Carb = carbohydrate. *n* = number of batches sampled during the experiment. Diets differed in their $\delta^{15}\text{N}$ ($F_{3,20} = 283.25$, $P < 0.0005$), %N ($F_{3,20} = 543.65$, $P < 0.0005$), $\delta^{13}\text{C}$ ($F_{3,20} = 2,291.28$, $P < 0.0005$), and %C ($F_{3,20} = 22.02$, $P < 0.0005$). Percent C and percent N were arcsine transformed before analysis. Within a column, values that do not share the same superscript are significantly different from one another ($P < 0.05$).

effect of waxworms on the isotopic composition of birds, we captured 10 birds during fall 2002 and fed them the same C₃ diet as birds captured during 2001, but these birds were not fed waxworms. These 10 birds maintained mass throughout the experiment. All birds from both years were weighed every day and provided new food at 0800 hours. All animal husbandry and care during this experiment were approved by the University of Rhode Island Institutional Animal Care and Use Committee (A98-09-012).

After birds were fed these diets for 2.5 mo, we sampled approximately 70 μL of blood at 1700 hours on day 0 from the brachial vein of six birds fed the C₃ diet, five birds fed the

C₄ diet, and 10 birds fed the C₃ diet without waxworms (C₃ w/o worms). Blood samples in capillary tubes were centrifuged within 1 h of collection, and the plasma and red blood cells (RBC) were transferred to separate cryovials and then stored frozen in liquid nitrogen. Birds were killed and then frozen immediately after blood collection. We later removed the pectoralis, liver, and small intestine from all birds (C₃: $n = 6$; C₄: $n = 6$; C₃ w/o worms: $n = 10$).

At 0800 hours on day 1, we switched the diet of the remaining birds (C₃: $n = 28$; C₄: $n = 29$) from their original diet to one of five diets (Fig. 1). Four birds fed the C₃ acclimation diet were switched to the C₄ diet, and the remaining C₃ birds ($n = 24$) were switched to one of four experimental diets (Table 2). Likewise, five birds fed the C₄ acclimation diet were switched to the C₃ diet, and the remaining C₄ birds ($n = 24$) were switched to one of four experimental diets (Table 2; Fig. 1). Two of the experimental diets had the same macronutrient composition as the two acclimation diets, but the protein and carbohydrate had different isotopic signatures (Table 2). The two other experimental diets had less protein (4% vs. 25%) and more carbohydrate (74% vs. 53%), and the protein and carbohydrate had different isotopic signatures (Table 2). To produce diets with the same macronutrient composition but with different isotopic signatures for those macronutrients (Table 2), it was necessary to use different amounts of some ingredients. All batches of the 25% protein diets contained 315.1 g of beet sugar or corn sugar, 150 g of casein or 238.1 g of fish meal, 68.9 g of olive oil or 47.5 g of corn oil, and 1,800 g of water. All batches of the 4% protein diets contained 441 g of beet sugar or corn sugar, 24 g of casein or 38.1 g of fish meal, 68.9 g of olive oil or 65.6 g of corn oil, and 1,800 g of water. All diets also contained essential vitamins (0.25 g vitamins/100 g wet food; AIN-76 Vitamin Mix, ICN Biomedicals), salts (1.25 g salts/100 g wet food; Briggs-N Salt Mix, ICN Biomedicals), and water (75 g water/100 g wet food). All diets were agar based (1.25 g agar/100 g wet food; Afik et al. 1997; Frazer and McWilliams 2002).

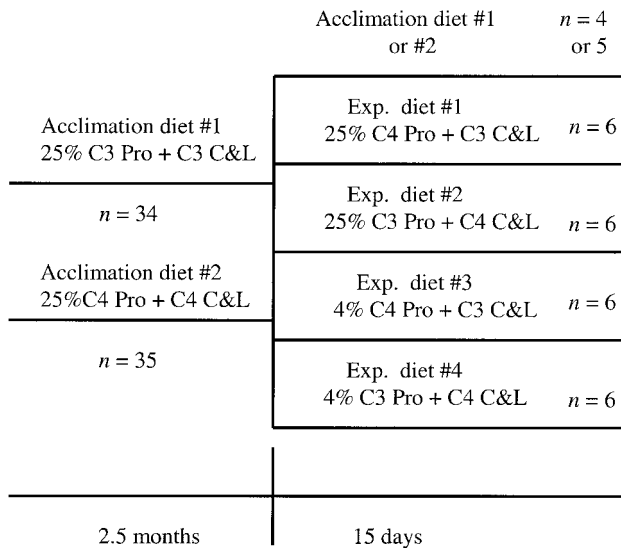


Figure 1. Flowchart of the experimental design illustrating the reciprocal nature of the experiment. Birds were initially fed either a C₃ protein + C₃ carbohydrate and lipid diet (acclimation diet 1) or a C₄ protein + C₄ carbohydrate and lipid diet (acclimation diet 2) for 2.5 mo. Birds were then switched to either the other acclimation diet or to one of four experimental diets for 15 d.

One bird switched from the C₃ to experimental diet 1 refused to eat the new diet and died on day 2, and one bird that was switched from the C₃ to C₄ diet was found dead in its cage on day 11 at 0800 hours. Cause of death is unknown. We collected blood samples from all remaining experimental birds on day 15 because this is approximately twice the stopover duration for songbirds on Block Island (Parrish 1997). We were unable to collect a blood sample from one bird switched from the C₄ acclimation diet to experimental diet 1. Birds were killed, and blood samples and carcasses were processed as described previously.

Isotopic Analysis

All samples were freeze-dried and powdered before isotopic analysis. Lipids were removed from the pectoralis, liver, and small intestine using petroleum ether as the solvent because tissues containing lipid are generally more depleted in ¹³C (DeNiro and Epstein 1977; Monson and Hayes 1980; Gannes et al. 1998). We extracted and recovered the lipid from the pectoralis muscle from six birds fed the C₃ diet for 2.5 mo and six birds fed the C₄ diet for 2.5 mo. We analyzed the pectoralis muscle with lipid and without lipid. We also analyzed the lipid extract from the pectoralis, and we analyzed a sample of the furcular fat from each one of these 12 birds (Table 3). Lipids were not removed from the plasma and RBC samples because there is generally a low proportion of lipid in blood (Bearhop et al. 2000, 2002). All tissue samples were analyzed at the Atlantic Ecology Division of the Environmental Protection Agency using a Carlo-Erba NA 1500 Series II Elemental Analyzer attached to a continuous-flow isotope ratio Micromass Optima spectrometer (CF-IRMS). Samples were converted to CO₂ and N₂ gases in oxidation/reduction furnaces and separated by gas chromatography. After separation, samples were measured for ¹³C/¹²C and ¹⁵N/¹⁴N ratios on the mass spectrometer. Stable isotope ratios are reported in δ-notation as parts per thousand (‰) deviations from Pee Dee belemnite (the international standard for C) for δ¹³C, and atmospheric N₂ for δ¹⁵N. Powdered dogfish muscle (DORM-1) reference material (National Research Council, Institute for Environmental Chem-

istry, Ottawa, Ontario) was used as a working standard. All samples were analyzed in duplicate. The same reference material analyzed over a several month period was measured with ± 0.3‰ precision. Percent C and N were calculated by deriving the masses of C and N in each sample by integrating the ion beam concentrations and comparing the integrals to standards with known concentrations of C and N and then dividing the mass of C and N in the sample by the sample weight and multiplying by 100. We report δ¹³C and δ¹⁵N for RBC, pectoralis muscle, liver, and small intestine, but only δ¹³C for plasma because small sample volume precluded measurement of δ¹⁵N in plasma.

Models and Statistical Analysis

To quantify differences between diet and tissue signatures for birds fed the acclimation diets for 2.5 mo, we calculated discrimination factors (Δ_{dt}) for each tissue using the equation: Δ_{dt} = δ_{tissue} - δ_{diet}. We used repeated-measures ANOVA to compare tissue signatures and discrimination factors between acclimation diets and to compare tissue signatures between treatments. If significant effects were found, we used one-way ANOVA with Bonferroni post hoc tests to determine differences within each diet or tissue. We also used one-way ANOVA with Bonferroni post hoc tests to compare isotopic signatures and percent composition of the six diets. For all statistical tests, P < 0.05 was deemed significant.

Results

Diet-Tissue Isotope Discrimination

As expected, δ¹³C and δ¹⁵N values of tissues from birds fed the C₄ acclimation diet were significantly more positive than birds fed the C₃ acclimation diet and birds fed the C₃ acclimation diet without waxworms (diet treatment: δ¹³C, F_{2,18} = 7,846.364, P < 0.0005; δ¹⁵N: F_{2,18} = 3,867.090, P < 0.0005). There were significant differences between tissues within each diet treatment (tissue: δ¹³C, F_{4,72} = 112.482, P < 0.0005; δ¹⁵N, F_{3,54} = 233.846, P < 0.0005; treatment × tissue: δ¹³C, F_{8,72} = 28.910, P < 0.0005; δ¹⁵N, F_{6,54} = 15.037, P < 0.0005; Fig. 2).

Table 3: δ¹³C values for pectoralis muscle, pectoralis muscle lipid, and furcular fat from yellow-rumped warblers fed the C₃ and C₄ acclimation diets for 2.5 mo

Acclimation Diet	δ ¹³ C (‰)				n
	Pectoralis without Lipid	Pectoralis with Lipid	Pectoralis Lipid	Furcular Fat	
C ₃	-22.9 ± .3 ^A	-23.9 ± .4 ^B	-27.6 ± .4 ^C	-27.2 ± .7 ^C	6
C ₄	-15.9 ± .1 ^A	-16.4 ± .2 ^{AB}	-18.7 ± .6 ^C	-16.9 ± .5 ^B	6

Note. Lipid composed 7.9% ± 3.0% of pectoral muscle of birds fed the C₃ diet and 10.0% ± 5.6% of birds fed the C₄ diet; n = number of birds sampled. Samples differed in their δ¹³C (repeated-measures ANOVA: C₃, F_{1,5} = 227.732, P < 0.0005; C₄, F_{1,5} = 90.413, P < 0.0005). Within a row, values that do not share the same superscript are significantly different from one another (P < 0.05).

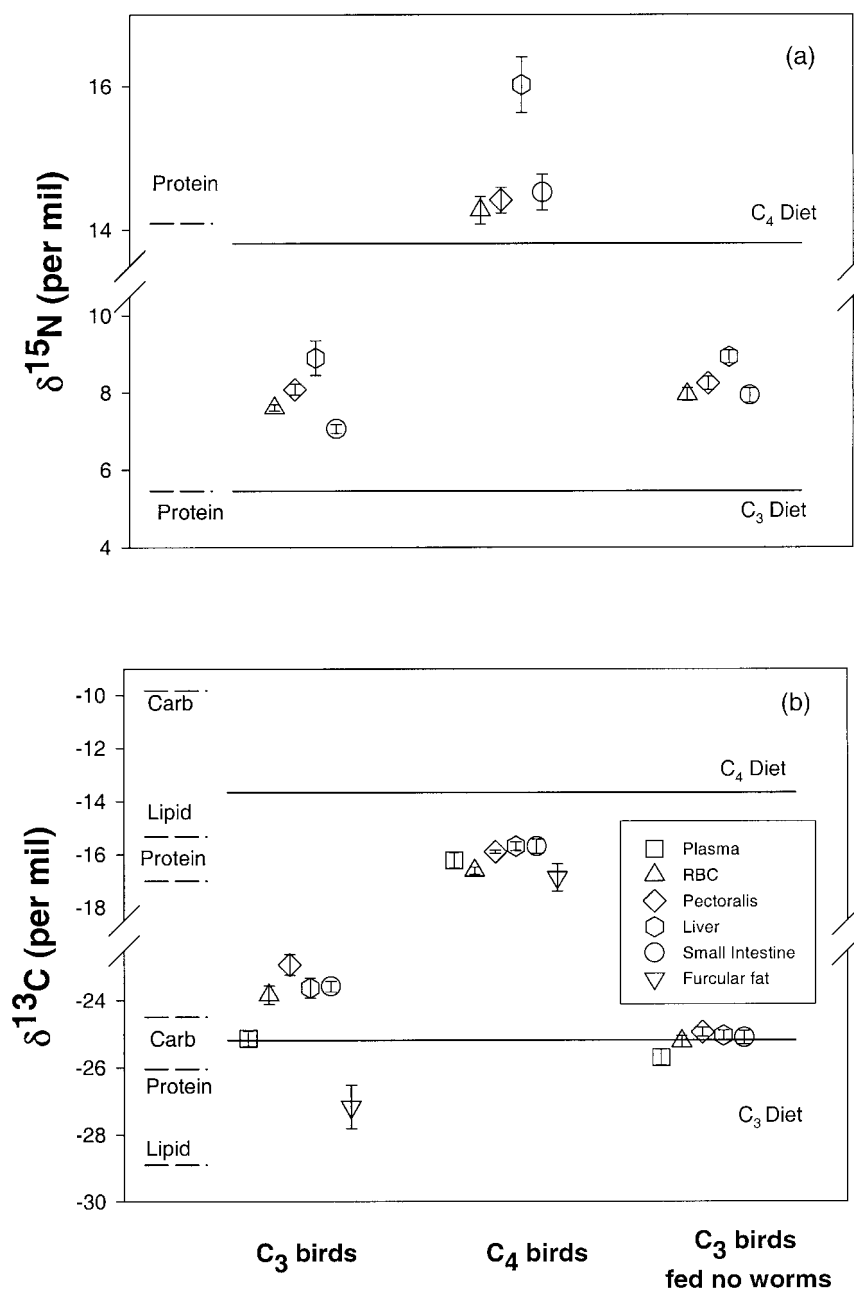


Figure 2. $\delta^{15}\text{N}$ (‰) (a) of red blood cells (RBC), pectoralis, liver, and small intestine, and $\delta^{13}\text{C}$ (‰) (b) of plasma, RBC, pectoralis, liver, small intestine, and furcular fat from yellow-rumped warblers fed the acclimation diets for 2.5 mo (mean \pm 1 SD; C_3 : $n = 6$; C_4 : $n = 6$; C_3 without waxworms: $n = 10$). Solid horizontal lines represent the $\delta^{15}\text{N}$ (a) and $\delta^{13}\text{C}$ (b) of the C_3 and C_4 bulk diets, and dotted horizontal lines represent the $\delta^{15}\text{N}$ (a) and $\delta^{13}\text{C}$ (b) of the relevant macronutrient components of the two diets.

There were only five blood samples for C_4 acclimation birds, and furcular fat was not part of this analysis because we did not analyze furcular fat for birds fed the C_3 acclimation diet without waxworms.

For birds fed the C_3 acclimation diet, $\delta^{15}\text{N}$ of RBC, pectoralis, liver, and small intestine were significantly different ($F_{3,20} =$

59.671, $P < 0.0005$), with liver the most enriched and small intestine on average the least enriched (Fig. 2a). $\delta^{15}\text{N}$ of liver was significantly more enriched than pectoralis, RBC, and small intestine for birds fed the C_4 acclimation diet ($F_{3,19} = 53.449$, $P < 0.0005$; Fig. 2a). Likewise, $\delta^{15}\text{N}$ of liver was significantly more enriched than pectoralis, RBC, and small intestine, and

RBC and small intestine were the least enriched for birds fed the C₃ acclimation diet without waxworms ($F_{3,36} = 69.627$, $P < 0.0005$; Fig. 2a).

For birds fed the C₃ acclimation diet, $\delta^{13}\text{C}$ of pectoralis was more enriched than all other tissues except for small intestine, and furcular fat was more depleted than all other tissues ($F_{5,30} = 112.899$, $P < 0.0005$; Fig. 2b). $\delta^{13}\text{C}$ of furcular fat was more depleted than plasma, pectoralis, liver, and small intestine but not RBC for birds fed the C₄ acclimation diet ($F_{5,28} = 20.067$, $P < 0.0005$; Fig. 2b). $\delta^{13}\text{C}$ of plasma was more depleted than RBC, pectoralis, liver, and small intestine for birds fed the C₃ acclimation diet without waxworms ($F_{4,45} = 25.787$, $P < 0.0005$; Fig. 2b).

Mean bulk-diet tissue discrimination factors ($\Delta\delta^{15}\text{N}$ and $\Delta\delta^{13}\text{C}$) were significantly different between birds fed a given acclimation diet (diet treatment: $\Delta\delta^{13}\text{C}$, $F_{2,18} = 1,039.231$, $P < 0.0005$; $\Delta\delta^{15}\text{N}$, $F_{2,18} = 255.869$, $P < 0.0005$), and there were significant differences between tissues within each diet treatment (tissue: $\Delta\delta^{13}\text{C}$, $F_{4,72} = 112.482$, $P < 0.0005$; $\Delta\delta^{15}\text{N}$, $F_{3,54} = 233.846$, $P < 0.0005$; treatment \times tissue: $\Delta\delta^{13}\text{C}$, $F_{8,72} = 28.910$, $P < 0.0005$; $\Delta\delta^{15}\text{N}$, $F_{6,54} = 15.037$, $P < 0.0005$; Table 4). In general, the liver had the greatest $\Delta\delta^{15}\text{N}$, and the small intestine and RBC had the smallest $\Delta\delta^{15}\text{N}$ for all three acclimation diets (Table 4). The $\Delta\delta^{13}\text{C}$ of birds fed the C₃ diet with waxworms was consistently more positive (range: 0.1–2.3) than that of birds fed the C₃ diet without waxworms (range: –0.5 to 0.2) and that of birds fed the C₄ diet (range: –2.0 to –3.0; Table 4).

Effects of Isotopic Signature and Concentration of Dietary Nutrients on Metabolic Routing

Carbon Routing. The greatest change in $\delta^{13}\text{C}$ of tissues for birds that had their diet switched occurred in birds that switched from their acclimation diet to the other acclimation diet, and

the least amount of change in $\delta^{13}\text{C}$ of tissues occurred in birds that switched to a low protein (4%) diet with the carbohydrate from the same source as their acclimation diet (diet 3 in Fig. 3; diet 4 in Fig. 4). In contrast, there was a significantly greater change in $\delta^{13}\text{C}$ of tissues in birds that switched to the 4% protein diet with the carbohydrate source that was different than the carbohydrate source in their acclimation diet (diet 4 in Fig. 3; diet 3 in Fig. 4). Also, change in $\delta^{13}\text{C}$ of high-protein tissues, such as pectoralis and small intestine, were similar for birds switched from their acclimation diet to either 25% protein diet (experimental diets 1 and 2; Figs. 3, 4), whereas $\delta^{13}\text{C}$ of plasma changed more in birds switched to the 25% protein diet with the carbohydrate signature the same as their acclimation diet (diet 2 in Fig. 3; diet 1 in Fig. 4).

We determined the relative contribution of dietary carbon from protein versus carbohydrate to final tissue signatures by comparing the amount of change in $\delta^{13}\text{C}$ of tissues between birds switched from their acclimation diet to the other acclimation diet with the amount of change in $\delta^{13}\text{C}$ of tissues in birds switched from their acclimation diet to the 25% or 4% protein diets with the protein source opposite to the protein source in their acclimation diet. For example, we compared birds switched from C₃ to C₄ with birds switched from C₃ to experiment diet 1 and birds switched from C₃ to experiment diet 3 (Fig. 5). We estimated that after 15 d approximately 50%–55% of the change in $\delta^{13}\text{C}$ of proteinaceous tissues of birds fed 25% protein diets came from carbon associated with dietary protein, and the remaining carbon came from other dietary sources. In contrast, only 10%–25% of the change in $\delta^{13}\text{C}$ of tissues in birds fed 4% protein diets came from carbon associated with dietary protein, and the remaining carbon came from other dietary sources (Fig. 5).

Nitrogen Routing. The greatest change in $\delta^{15}\text{N}$ of tissues for birds that had their diet switched occurred in birds that

Table 4: Mean bulk-diet tissue discrimination factors for yellow-rumped warblers fed the C₃ and C₄ acclimation diets (‰)

Acclimation Diet and Isotope	Plasma	RBC	Pectoralis	Liver	Small Intestine	F value	df	P value	n
C ₃ + worms:									
$\Delta\delta^{13}\text{C}$.1 ± .2 ^A	1.4 ± .3 ^B	2.3 ± .3 ^C	1.6 ± .3 ^B	1.6 ± .2 ^B	57.579	4, 25	<.0005	6
$\Delta\delta^{15}\text{N}$	NA	2.2 ± .1 ^A	2.6 ± .2 ^B	3.5 ± .5 ^C	1.6 ± .1 ^D	59.671	3, 20	<.0005	6
C ₃ without worms:									
$\Delta\delta^{13}\text{C}$	–.5 ± .2 ^A	.0 ± .2 ^B	.2 ± .1 ^C	.1 ± .2 ^{BC}	.1 ± .2 ^{BC}	25.787	4, 45	<.0005	10
$\Delta\delta^{15}\text{N}$	NA	2.5 ± .2 ^A	2.8 ± .2 ^B	3.5 ± .2 ^C	2.5 ± .2 ^A	69.627	3, 36	<.0005	10
C ₄ + worms:									
$\Delta\delta^{13}\text{C}$	–2.6 ± .3 ^{AB}	–3.0 ± .1 ^A	–2.2 ± .1 ^{BC}	–2.0 ± .2 ^C	–2.0 ± .3 ^C	20.388	4, 23	<.0005	6
$\Delta\delta^{15}\text{N}$	NA	.5 ± .2 ^A	.7 ± .2 ^A	2.3 ± .4 ^B	.8 ± .3 ^A	53.449	3, 19	<.0005	6

Note. Bulk-diet tissue discrimination factors ($\Delta\delta^{15}\text{N}$ and $\Delta\delta^{13}\text{C}$; mean ± 1 SD) for plasma, RBC, pectoralis, liver, and small intestine, calculated for yellow-rumped warblers fed the C₃ and C₄ acclimation diets for 2.5 mo and for yellow-rumped warblers fed the C₃ acclimation diet without waxworms for 2.5 mo. There were five blood samples collected from C₄ birds and six samples collected for all other tissues. F and P values are for one-factor ANOVA. Within a row, values that do not share the same superscript are significantly different from one another ($P < 0.05$). n = number of birds sampled; NA = not available.

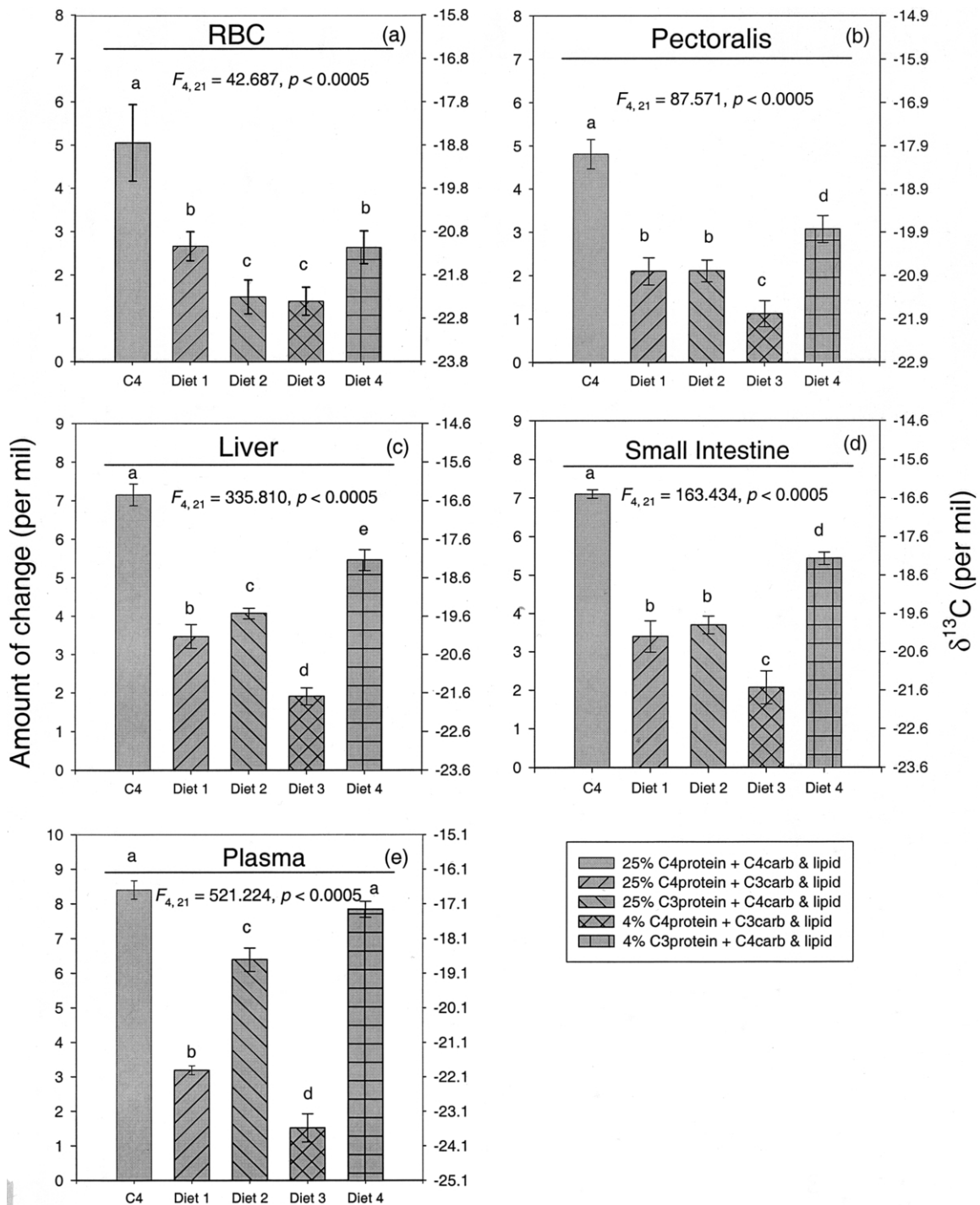


Figure 3. Absolute $\delta^{13}\text{C}$ (‰; mean ± 1 SD; right Y-axes) and relative amount of change in $\delta^{13}\text{C}$ (‰; left Y-axes) in RBC (a), pectoralis (b), liver (c), small intestine (d), and plasma (e) for yellow-rumped warblers fed the C_3 acclimation diet for 2.5 mo and then switched for 15 d to either the C_4 diet or diets 1–4. X-axis in each panel intersects the Y-axis at the mean $\delta^{13}\text{C}$ (‰) for birds fed the C_3 acclimation diet for 2.5 mo. Solid horizontal line is the mean $\delta^{13}\text{C}$ (‰) for birds fed the C_4 acclimation diet for 2.5 mo for each tissue; $n = 6$ birds for all samples except C_4 ($n = 3$ birds) and diet 1 ($n = 5$ birds). Bars with the same letter above are not significantly different (diet treatment: $F_{4,21} = 477.618, P < 0.0005$; tissue: $F_{4,84} = 186.374, P < 0.0005$; diet \times tissue: $F_{16,84} = 52.836, P < 0.0005$).

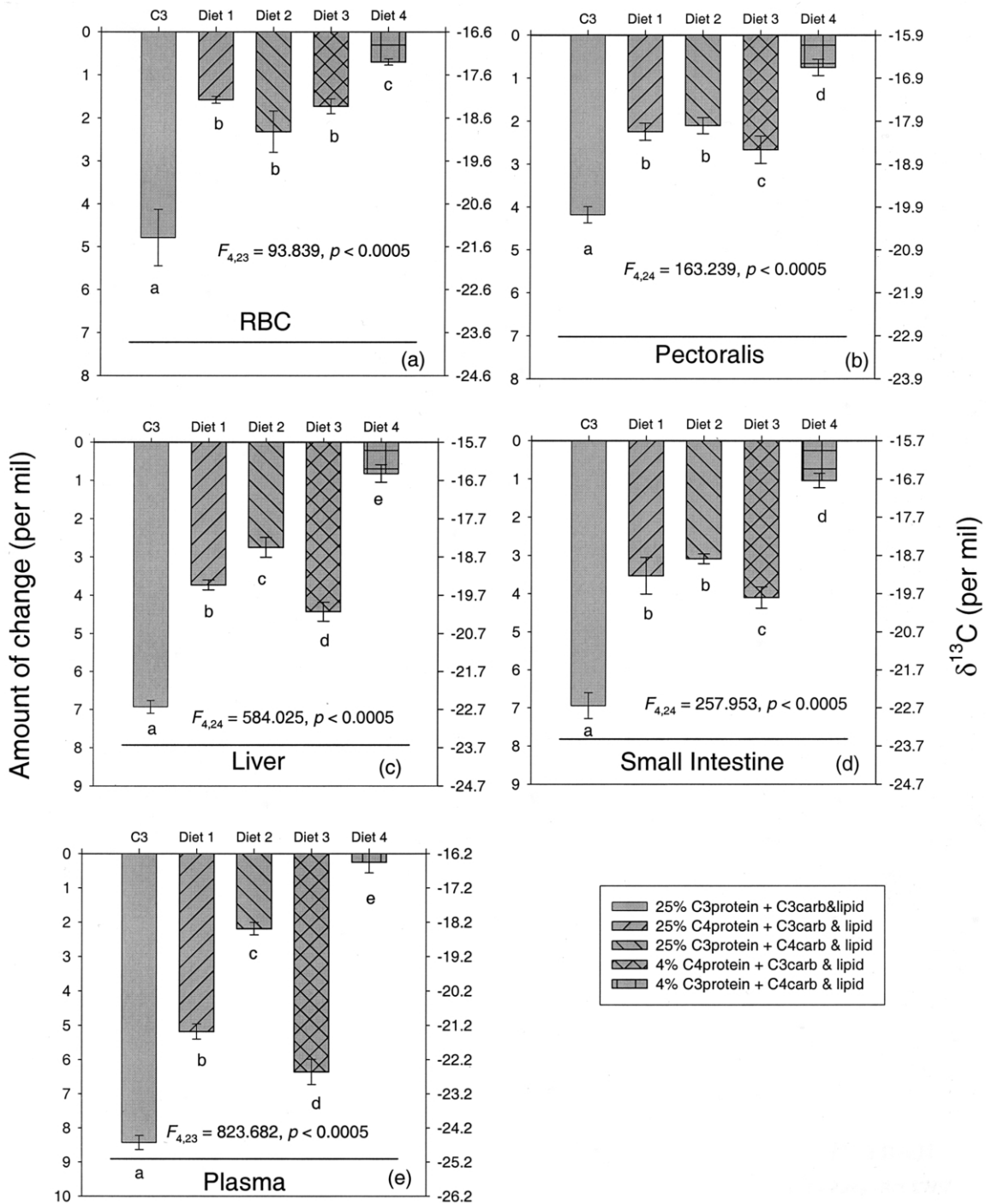


Figure 4. Absolute $\delta^{13}\text{C}$ (‰; mean \pm 1 SD; right Y-axes) and relative amount of change in $\delta^{13}\text{C}$ (‰; left Y-axes) in RBC (a), pectoralis (b), liver (c), small intestine (d), and plasma (e) for yellow-rumped warblers fed the C₄ acclimation diet for 2.5 mo and then switched for 15 d to either the C₃ diet or diets 1–4. X-axis in each panel intersects the Y-axis at the mean $\delta^{13}\text{C}$ (‰) for birds fed the C₄ acclimation diet for 2.5 mo. Solid horizontal line is the mean $\delta^{13}\text{C}$ (‰) for birds fed the C₃ acclimation diet for 2.5 mo for each tissue; $n = 6$ birds for all samples, except C₃ ($n = 5$ birds) and diet 1; RBC and plasma ($n = 5$ birds). Bars with the same letter below are not significantly different (diet treatment: $F_{4,23} = 661.323, P < 0.0005$; tissue: $F_{4,92} = 443.637, P < 0.0005$; diet \times tissue: $F_{16,92} = 104.856, P < 0.0005$).

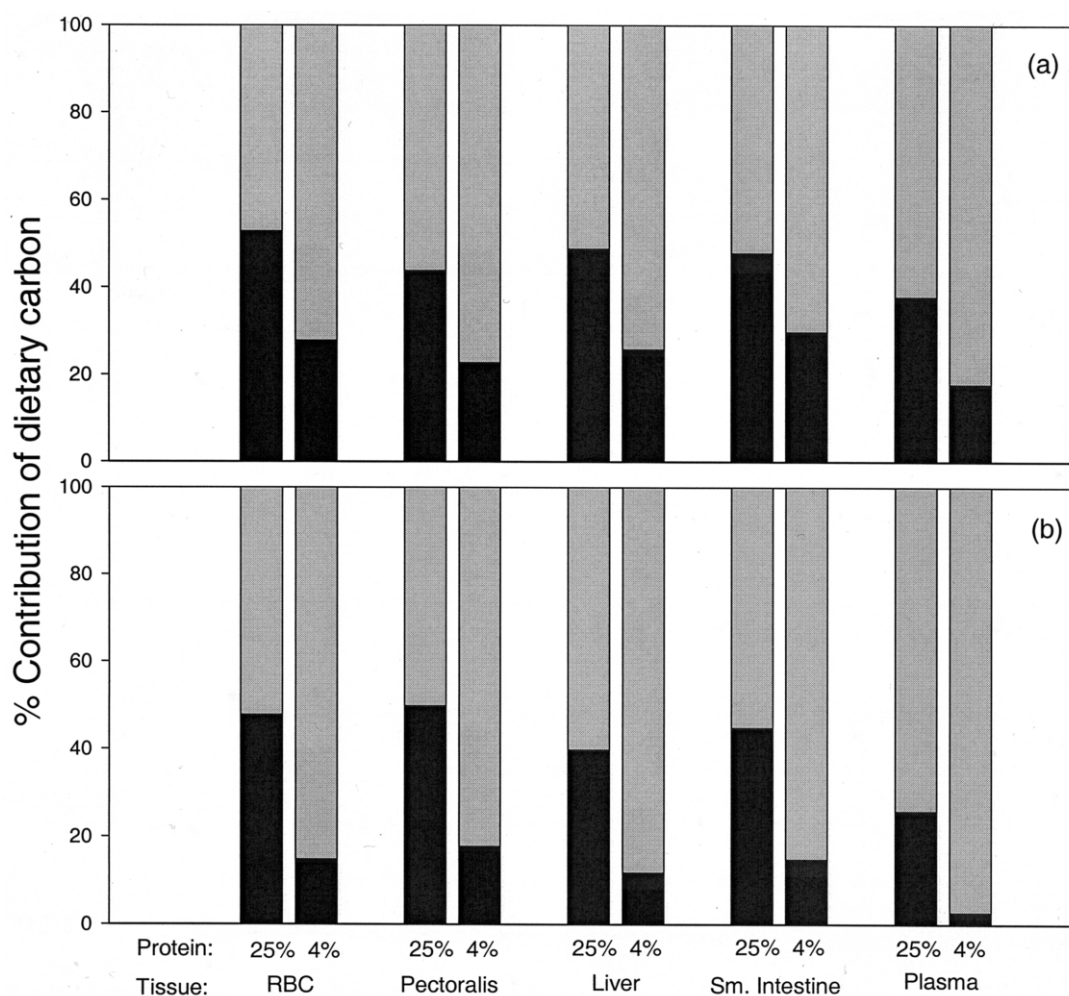


Figure 5. Relative contribution of dietary carbon to tissue carbon for yellow-rumped warblers switched 15 d ago from the C₃ acclimation diet (a) and for birds switched 15 d ago from the C₄ acclimation diet (b). Information compiled from Figures 3 and 4. Black represents dietary protein contribution to tissue carbon, and gray represents nonprotein carbon contributions to tissue carbon.

switched to the other acclimation diet and for birds switched to the experimental diet, with 25% protein from the opposite source as their acclimation diet (C₄ and diet 1 in Fig. 6; C₃ and diet 2 in Fig. 7). In general, $\delta^{15}\text{N}$ of tissues from birds fed the 4% protein diets containing protein from the opposite protein source as their acclimation diet changed less than that of birds switched to the 25% protein diets (diet 3 in Fig. 6; diet 4 in Fig. 7). Less change in $\delta^{15}\text{N}$ of tissues occurred in birds that switched from their acclimation diet to diets composed of the same protein source as their acclimation diets (C₃ birds switched to diets 2 and 4, and C₄ birds switched to diets 1 and 3; Figs. 6, 7).

We determined the relative contribution of dietary protein to final tissue signatures by comparing the amount of change in $\delta^{15}\text{N}$ of tissues between birds switched from their acclimation diet to the other acclimation diet with the amount of

change in $\delta^{15}\text{N}$ of tissues in birds switched from their acclimation diet to the 25% and 4% protein diets, with the protein source opposite to the protein source in their acclimation diet. For example, we compared birds switched from C₃ to C₄ with birds switched from C₃ to experimental diet 1 and birds switched from C₃ to experimental diet 3 (Fig. 8). We estimated that after 15 d almost 100% of the change in $\delta^{15}\text{N}$ of proteinaceous tissues of birds fed 25% protein diets came from nitrogen associated with dietary protein. However, birds fed 4% protein had less change in $\delta^{15}\text{N}$ of proteinaceous tissues after 15 d, and approximately 20%–80% of the nitrogen in proteinaceous tissues was routed directly from dietary protein (Fig. 8). Also, birds acclimated to the C₄ diet and switched to a low-protein diet had a greater change in $\delta^{15}\text{N}$ of tissues than birds acclimated to the C₃ diet switched to a low-protein diet (Fig. 8).

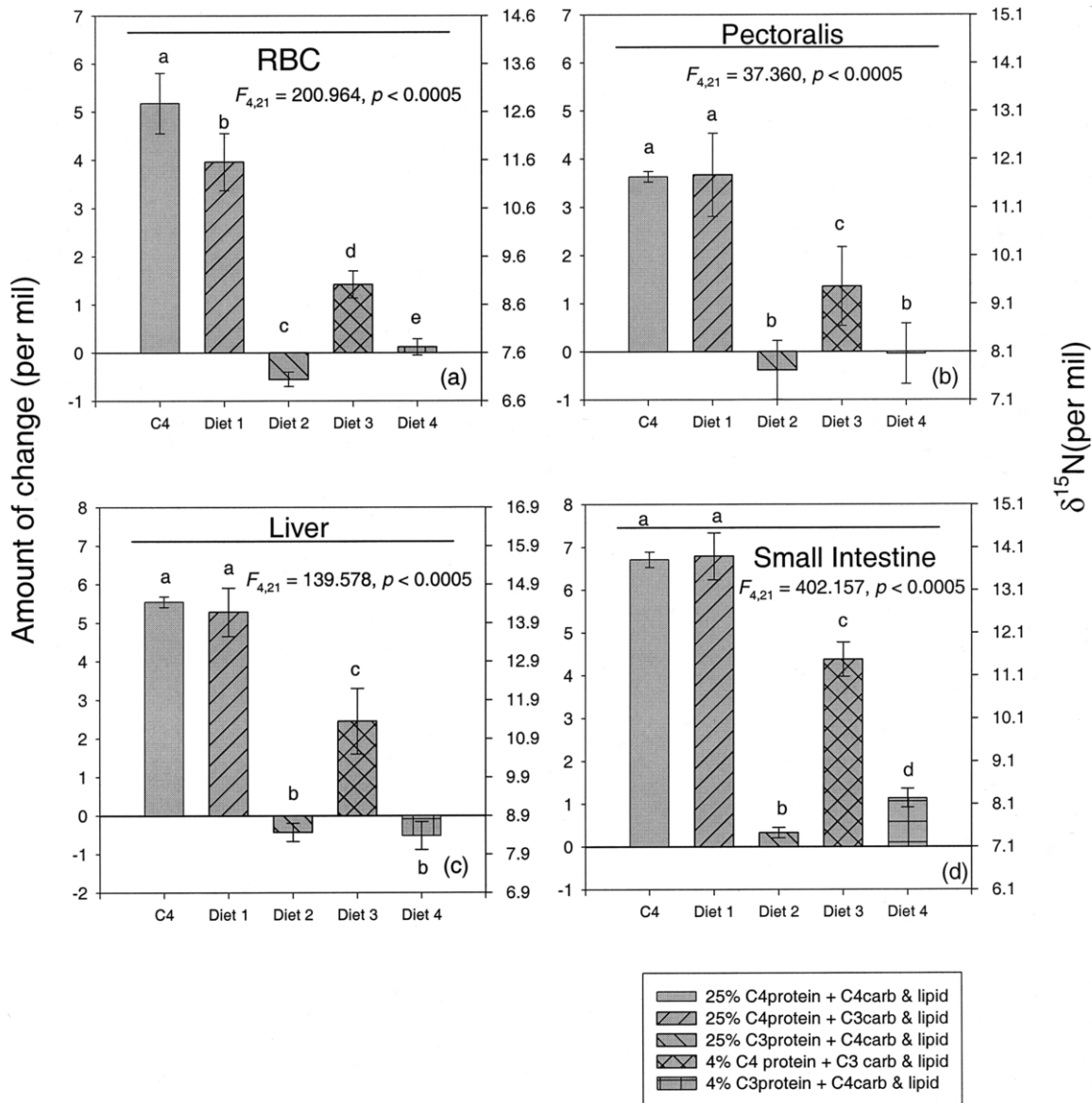


Figure 6. Absolute $\delta^{15}\text{N}$ (‰; mean \pm 1 SD; right Y-axes) and relative amount of change in $\delta^{15}\text{N}$ (‰; left Y-axes) in RBC (a), pectoralis (b), liver (c), and small intestine (d) for yellow-rumped warblers fed the C_3 acclimation diet for 2.5 mo and then switched for 15 d to either the C_4 diet or diets 1–4. X-axis in each panel intersects the Y-axis at the mean $\delta^{15}\text{N}$ (‰) for birds fed the C_3 acclimation diet for 2.5 mo. Solid horizontal line is the mean $\delta^{15}\text{N}$ (‰) for birds fed the C_4 acclimation diet for 2.5 mo for each tissue; $n = 6$ birds for all samples except C_4 ($n = 3$ birds) and experimental diet 1 ($n = 5$ birds). Bars with the same letter above are not significantly different (diet treatment: $F_{4,21} = 275.317, P < 0.0005$; tissue: $F_{3,63} = 95.442, P < 0.0005$; diet \times tissue: $F_{12,63} = 10.729, P < 0.0005$).

Discussion

Diet-Tissue Isotope Discrimination

Discrimination factors for carbon and nitrogen are commonly used by ecologists to construct trophic relationships (Hobson et al. 2000; Post 2002) and to reconstruct the contributions of multiple food components to an animal's diet (Ben-David et al. 1997). Typically, discrimination factors are calculated as the

difference between bulk-diet and tissue signature. Our results demonstrate that diets with the same macronutrient composition but different macronutrient signatures can produce different discrimination factors. In our study, a more accurate estimate of discrimination factors was obtained for animal tissues that were primarily protein if calculated as the difference between the signature of the dietary protein and the tissue.

Bulk-diet nitrogen discrimination factors ($\Delta\delta^{15}\text{N}$) for yellow-

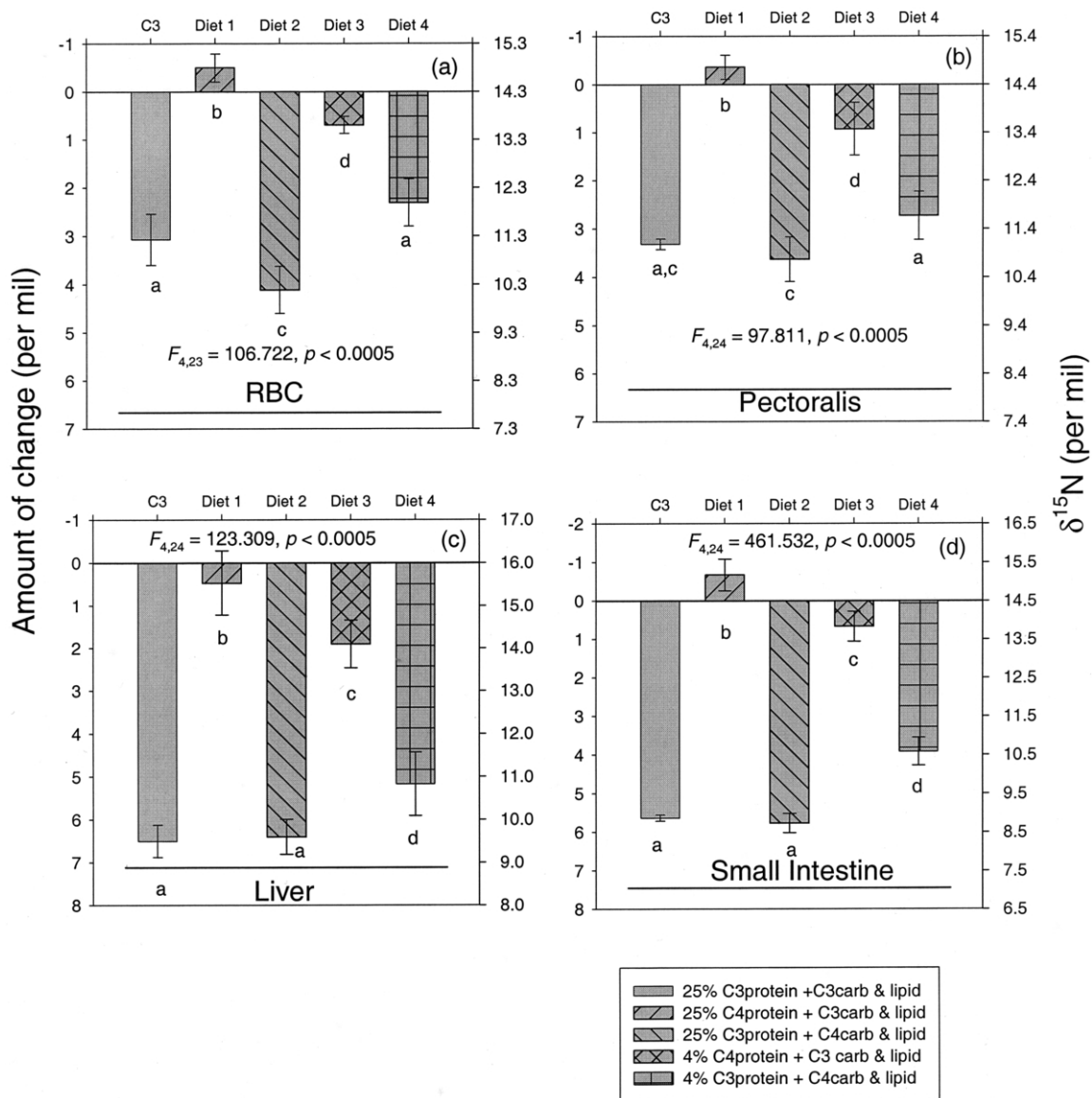


Figure 7. Absolute $\delta^{15}\text{N}$ (‰; mean \pm 1 SD; right Y-axes) and relative amount of change (‰; left Y-axes) in RBC (a), pectoralis (b), liver (c), and small intestine (d) for yellow-rumped warblers fed the C₄ acclimation diet for 2.5 mo and then switched for 15 d to either the C₃ diet or diets 1–4. X-axis in each panel intersects the Y-axis at the mean $\delta^{15}\text{N}$ (‰) for birds fed the C₄ acclimation diet for 2.5 mo. Solid horizontal line is the mean $\delta^{15}\text{N}$ (‰) for birds fed the C₃ acclimation diet for 2.5 mo for each tissue; $n = 6$ birds for all samples except C₃ ($n = 5$ birds) and experimental diet 1 (RBC: $n = 5$ birds). Bars with the same letter below are not significantly different (diet treatment: $F_{4,23} = 431.139, P < 0.0005$; tissue: $F_{3,69} = 25.822, P < 0.0005$; diet \times tissue: $F_{12,69} = 13.452, P < 0.0005$).

rumped warblers fed the three acclimation diets were within the range reported by other authors (DeNiro and Epstein 1981; Hobson and Clark 1992b; Hobson and Bairlein 2003; Pearson et al. 2003). In our study, $\Delta\delta^{15}\text{N}$ ranged from $0.5\text{‰} \pm 0.2\text{‰}$ in RBC of C₄ birds to $3.5\text{‰} \pm 0.2\text{‰}$ in liver of C₃ birds fed no worms (Table 4). Waxworms had little influence on $\delta^{15}\text{N}$ discrimination factors for birds fed the C₃ acclimation diet because $\delta^{15}\text{N}$ of the waxworm (7.0‰) was similar to the $\delta^{15}\text{N}$

of the protein source of the C₃ diet ($5.9\text{‰} \pm 0.8\text{‰}$; Table 4). $\Delta\delta^{15}\text{N}$ for all tissues was less overall for C₄ birds compared with birds fed the C₃ acclimation diet with and without waxworms (Table 4). One potential reason for the smaller $\Delta\delta^{15}\text{N}$ for C₄ birds was that the waxworm was considerably less enriched than the protein source of the C₄ diet ($14.1\text{‰} \pm 0.1\text{‰}$). For birds in the wild that consume primarily fruit but supplement opportunistically with insects, the insect portion of the diet

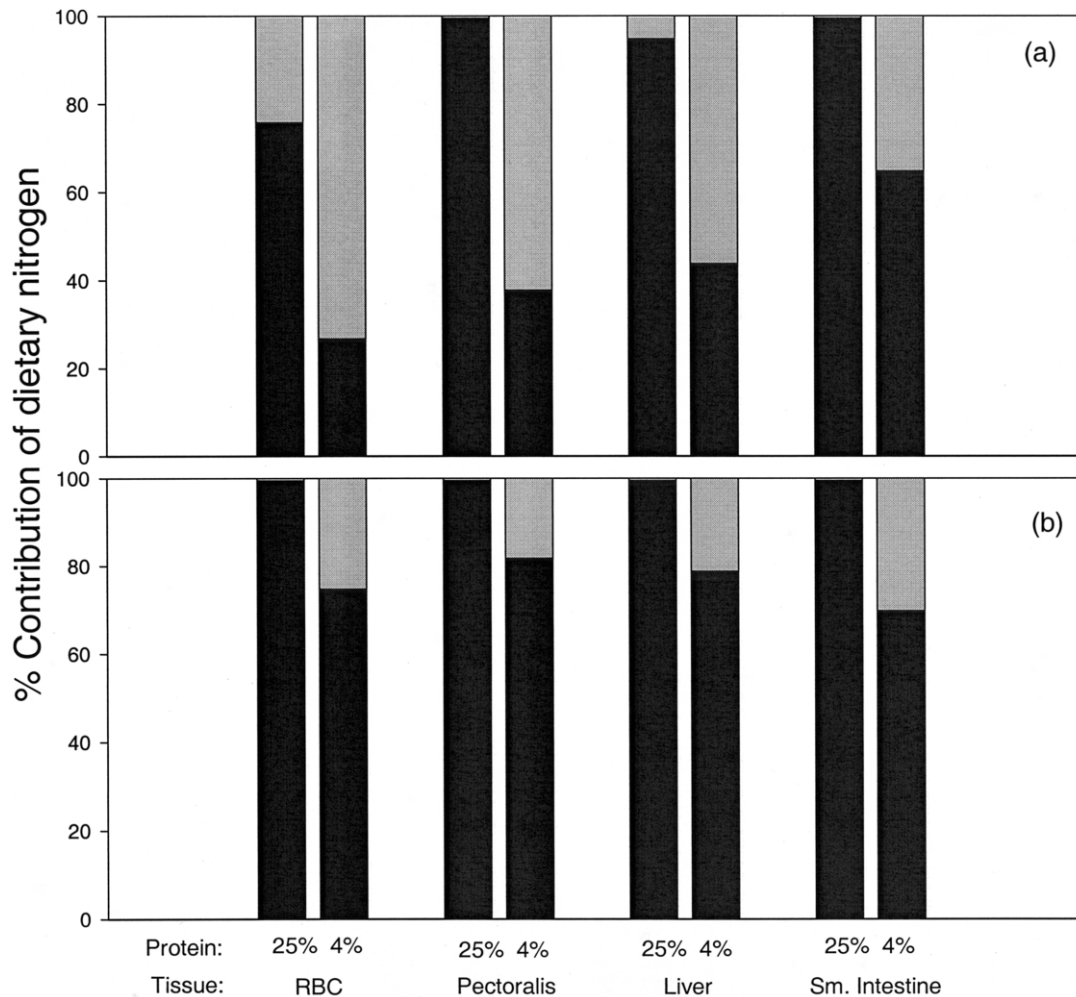


Figure 8. Relative contribution of dietary nitrogen to tissue nitrogen for yellow-rumped warblers switched 15 d ago from the C₃ acclimation diet (a) and for birds switched 15 d ago from the C₄ acclimation diet (b). Information compiled from Figures 6 and 7. Black represents dietary protein contribution to tissue signature, and gray represents relative amount of tissue signature unchanged by dietary protein.

could also have a disproportionate influence on $\delta^{15}\text{N}$ tissue values if $\delta^{15}\text{N}$ of the insect protein and $\delta^{15}\text{N}$ of the fruit protein are quite different, as in our C₄ diet with waxworms.

Bulk-diet tissue carbon discrimination factors ($\Delta\delta^{13}\text{C}$) ranged from $-3.0\text{‰} \pm 0.1\text{‰}$ for RBC of C₄ birds to $2.3\text{‰} \pm 0.3\text{‰}$ for pectoralis of C₃ birds fed waxworms (Table 4). This range in diet discrimination factors for $\delta^{13}\text{C}$ is generally broader than reported by other researchers (DeNiro and Epstein 1978; Tieszen et al. 1983; Mizutani et al. 1992), although Pearson et al. (2003) calculated discrimination factors for yellow-rumped warblers that ranged from -1.2‰ to 2.2‰ for whole blood and 1.9‰ to 4.3‰ for feathers, depending on diet composition. Comparison of carbon discrimination factors for birds fed the C₃ diet with and without waxworms indicated that the waxworm increased the difference between bulk-diet and tissue $\delta^{13}\text{C}$ of

the waxworm (-20.8‰) was more enriched than the C₃ diet and diet macronutrients (Table 4). Birds fed the C₄ acclimation diet had unusually negative discrimination factors (Table 4). Two possible explanations for such negative carbon discrimination factors for birds fed the C₄ diet are that both the protein source for the C₄ diet and the protein in the waxworm had more depleted signatures than the C₄ bulk diet. If discrimination factors for C₄ birds are calculated between dietary protein and tissue instead of bulk diet and tissue, the range in discrimination factors (0.4‰ – 1.3‰) is more similar to other published estimates of bulk-diet discrimination factors (DeNiro and Epstein 1978; Hobson and Clark 1992a; Mizutani et al. 1992). Therefore, accurate estimates of tissue carbon discrimination factors should be calculated between the tissue and the macronutrient most used in tissue synthesis.

Estimating the Metabolic Routing of Dietary Nutrients

Testing Hypothesis 1: Dietary Protein Routes Metabolically to Tissue Protein. We hypothesized that the carbon and nitrogen signatures of proteinaceous tissue in birds fed nutritionally adequate diets would have isotopic signatures similar to dietary protein. We tested this hypothesis by comparing the observed $\delta^{13}\text{C}$ of tissues from birds fed the acclimation diets for 2.5 mo and birds switched to new diets for 15 d with the predicted $\delta^{13}\text{C}$ of tissues from concentration-dependent mixing models (Phillips 2001; Phillips and Koch 2002; Podlesak 2004; Martínez del Rio and Wolf 2005). We did not model tissue nitrogen signatures in this same way because carbohydrate and lipid contain minuscule amounts of nitrogen so that dietary protein supplied the only source of exogenous nitrogen.

We used the mixing models to predict $\delta^{13}\text{C}$ for proteinaceous tissue given the carbon signatures of the dietary macronutrients and assuming 0%–100% of the dietary protein contributed to tissue synthesis. We then compared the predicted and observed $\delta^{13}\text{C}$ in tissues to estimate the relative contribution of each dietary macronutrient to tissue carbon. For each of the three acclimation groups, we created two models: one model assumed that birds used protein, carbohydrate, and lipid to synthesize tissue protein, and the other model assumed that the bird only used dietary protein and carbohydrate for tissue synthesis (Fig. 9). Models developed for the diet groups that were supplemented with waxworms assumed that birds received approximately 20% of daily protein intake and approximately 12% of nonprotein carbon intake from the waxworm. In this formulation of the models, we assumed that all dietary protein assimilated equally, and we did not add a discrimination factor to the models because there was $<1\text{‰}$ diet-tissue discrimination for birds fed the C_3 diet without waxworms (Table 4). We did not vary these parameters; therefore, these models are insensitive to potential variations in the above assumptions.

If birds fed nutritionally adequate diets exclusively route dietary protein into protein synthesis, our models predicted that dietary protein supplies approximately 0%–100% of carbon for tissue protein in birds fed the C_3 diet with or without waxworms and 75%–90% of carbon for protein in birds fed the C_4 diet (Fig. 9). However, because each acclimation diet was composed of ingredients from one pathway (either C_3 or C_4), whether carbon was routed from dietary protein, carbohydrate, or lipid into tissue synthesis was unclear.

Results from the diet-switching experiments provide a clearer picture of dietary routing of macronutrients because the dietary macronutrient signatures are by design more distinct. We predicted $\delta^{13}\text{C}$ of two proteinaceous tissues, blood plasma, and liver for birds in the diet-switching experiments using the same model as above (Fig. 10). Although we sampled plasma and liver 15 d after the diet switch, at least 90% of carbon in plasma and liver of yellow-rumped warblers should have turned over by this time (Pearson et al. 2003; Podlesak et al. 2005). Our

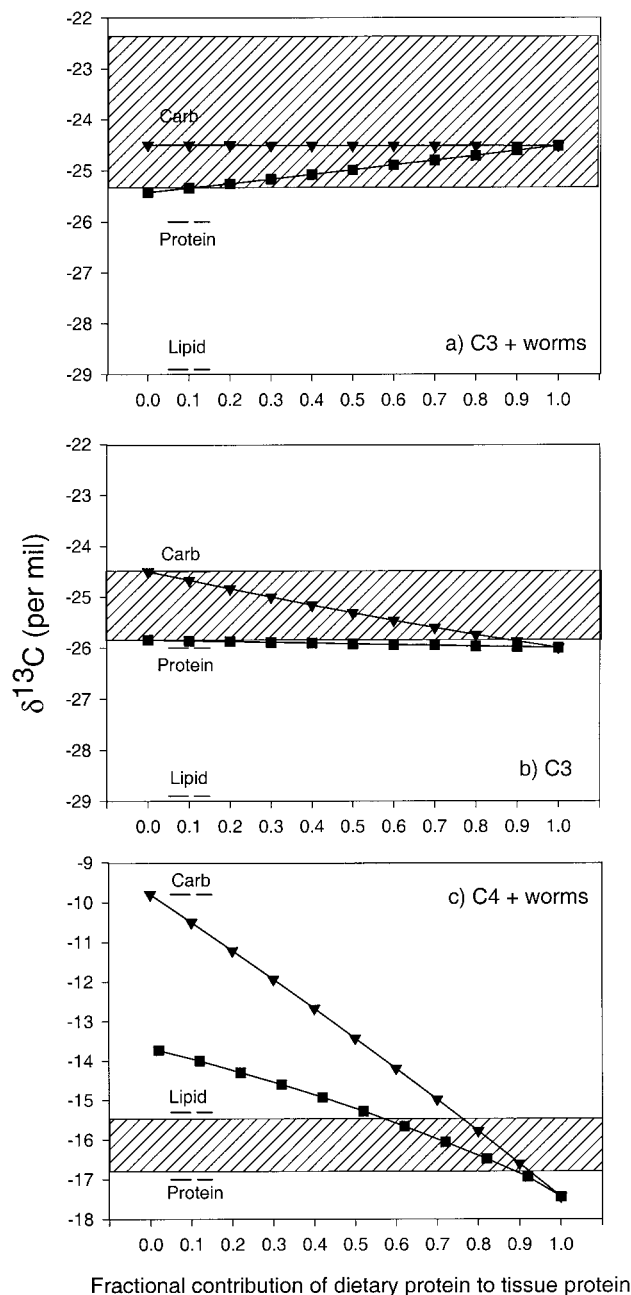


Figure 9. Observed (shaded areas) and predicted (lines) $\delta^{13}\text{C}$ of proteinaceous tissues for birds fed the C_3 diet with waxworms (a), for birds fed the C_3 diet without waxworms (b), and for birds fed the C_4 diet with waxworms (c). X-axes are the percent contribution of dietary protein to tissue protein. Predicted $\delta^{13}\text{C}$ for one concentration-dependent mixing model (solid lines with squares) assumes birds use carbon from dietary protein, carbohydrate, and lipid to synthesize protein. Predicted $\delta^{13}\text{C}$ for the other concentration-dependent mixing model (solid lines with triangles) assumes birds use only dietary protein and carbohydrate to synthesize protein. Shaded area in each panel is the range in $\delta^{13}\text{C}$ for all proteinaceous tissues sampled from captive birds; short dashed lines are $\delta^{13}\text{C}$ of dietary macronutrients.

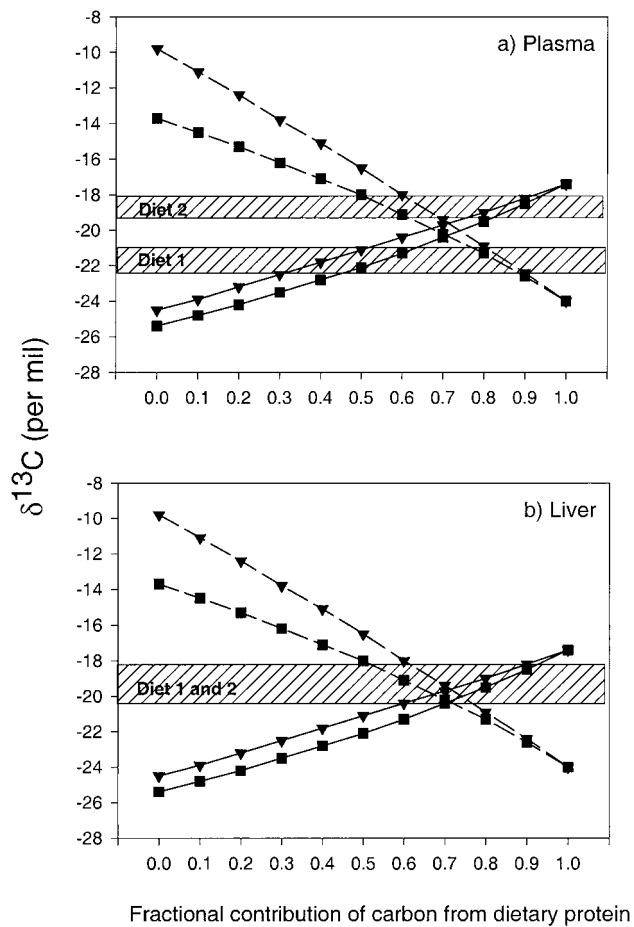


Figure 10. Observed (shaded areas) and predicted (lines) $\delta^{13}\text{C}$ of proteinaceous tissues for birds fed adequate protein diets with the protein labeled differently than the carbohydrate and lipid. Solid lines represent 25% C_4 protein + C_3 carbohydrate and lipid (diet 1) and dotted lines represent 25% C_3 protein + C_4 carbohydrate and lipid (diet 2). X-axes are the percent contribution of dietary protein to tissue protein. Predicted $\delta^{13}\text{C}$ for one concentration-dependent mixing model (lines with squares) assumes birds use carbon from dietary protein, carbohydrate, and lipid to synthesize protein. Predicted $\delta^{13}\text{C}$ for the concentration-dependent mixing model (lines with triangles) assumes birds use only dietary protein and carbohydrate to synthesize protein. Shaded area in each panel is the range in $\delta^{13}\text{C}$ for plasma (a) and liver (b) sampled from captive birds fed diets 1 and 2 for 15 d.

models predicted dramatic differences in tissue signature, depending on the amount of routing from nonprotein sources of carbon into proteinaceous tissue (Fig. 10). We estimated that dietary carbohydrate and/or lipid supplies 30%–60% of the carbon in plasma and supplies 50%–95% of the carbon in liver (Fig. 10). This evidence combined with the evidence from the models suggests that birds metabolically route carbon from nonprotein dietary sources into proteinaceous tissue synthesis.

Our experiment was designed to investigate the metabolic routing of carbon and nitrogen 15 d after a change in diet. We

could have instead sampled birds 75 d after the diet switch (tissues in isotopic equilibrium with diet), but we were interested in investigating the effect that differing levels of dietary protein may have on tissue signatures over a time period similar to the amount of time wild songbirds spend at stopover sites. Granted, such an experimental design may have certain limitations as to the ability to extrapolate conclusions to other animal systems; however, our results are directly applicable to other small animals with similar rapid rates of tissue turnover. We concluded that small songbirds route carbon from nonprotein sources into tissue synthesis during the 15 d after a change in diet. It remains to be determined whether songbirds continue to metabolically route carbon from nonprotein dietary sources into proteinaceous tissue for longer than 15 d after a diet switch.

The results from the concentration-dependent mixing models do not support the hypothesis that carbon from dietary protein routes directly to tissue protein. Likewise, we estimated that dietary protein contributed only 50%–55% of carbon in tissue protein from dietary protein based on our comparisons between birds switched to the experimental diets (Fig. 5), whereas almost 100% of the change in $\delta^{15}\text{N}$ of the proteinaceous tissues of birds fed adequate protein diets was attributable to dietary protein (Fig. 8). Thus, the results from the concentration-dependent models combined with the results from the diet-switching experiment indicate that there is a decoupling of carbon and nitrogen routing into protein synthesis for birds fed diets with adequate protein. This result is different than expected and is also different than that found by other researchers (Hilderbrand et al. 1996; Haramis et al. 2001; Bearhop et al. 2002; Evans-Ogden et al. 2004). However, this result does support the hypothesis that the assimilation and routing of carbon and nitrogen may differ depending on diet composition (Bearhop et al. 2002; Pearson et al. 2003). As a result, $\delta^{15}\text{N}$ of tissue samples collected from wild animals that consume high-quality diets may reliably indicate the dietary protein source, whereas $\delta^{13}\text{C}$ of these same tissues is likely the product of metabolic routing of carbon from several macronutrients.

Testing Hypothesis 2: Birds Route Carbon from Nonprotein Dietary Sources into Protein Synthesis. We also hypothesized that birds fed diets with less protein must metabolically route carbon from nonprotein dietary sources into protein synthesis. We estimated that dietary protein contributed 10%–25% of the carbon in tissue protein of birds switched to the low-protein diets and that dietary carbohydrate and/or lipid contributed the remainder (Fig. 5). In some cases, there was as much or more change in $\delta^{13}\text{C}$ of tissues for birds fed the low-protein diets compared with birds fed the 25% protein diets (Figs. 3, 4).

The magnitude of change in $\delta^{15}\text{N}$ of tissues was dependent on the protein concentration of the diet and the isotopic signature of the dietary protein. Birds that were switched to a diet

with the same protein source as their acclimation diet had little or no change in $\delta^{15}\text{N}$ of their tissues. Conversely, birds that were switched to diets with the protein from the opposite source as their acclimation diet had the greatest changes in $\delta^{15}\text{N}$ values for birds that were fed the higher-protein diet. Thus, there is also a decoupling of carbon and nitrogen routing into protein synthesis for birds fed low-protein diets. As a consequence, tissue carbon signatures would most likely indicate a change in diet before tissue nitrogen signatures for birds that change diets to low-protein/high-carbohydrate fruits.

Caveats and Future Directions

It is clear from our research and the research of others that variations in concentration and isotopic signatures of the macronutrients within a diet can have significant effects on the isotopic signatures of tissues in animals (Pearson et al. 2003). If the macronutrients within the diets of wild animals have relatively unique isotopic signatures, then it should be possible to determine how these macronutrients are routed into an animal's tissues. For example, during migration many songbirds eat primarily fruit but supplement opportunistically with insects. Fruits and insects differ nutritionally, with insects higher in protein and lower in carbohydrates than fruits. Concentration-dependent mixing models that incorporate the nutritional composition and the isotopic concentration of the fruits and insects in the diet of songbirds could determine the importance of each dietary component to tissue synthesis.

However, at this time we know little about the isotopic signatures of the bulk diets of wild animals and less about the isotopic signatures and composition of the macronutrients within the diets of wild animals. Because little is known about the isotopic composition of the diets of wild animals, most diet reconstruction using stable isotopes is based on the difference between the isotopic signatures of the bulk-diet and specific tissues. Because different tissues within the animal turn over at different rates and animals metabolically route macronutrients from multiple dietary sources into tissue (i.e., carbon from dietary protein and dietary carbohydrate into proteinaceous tissue), such techniques may under- or overestimate the relative importance of one diet component. Therefore, stable isotope analysis of the macronutrients within the major dietary components of an animal's diet combined with estimates of the effects of diet composition on tissue signature provides a more accurate method to determine the relative importance of a given food source(s) for satisfying an animal's nutritional requirements.

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