ECOPHYSIOLOGY

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Stable isotopes in breath, blood, feces and feathers can indicate intra-individual changes in the diet of migratory songbirds

Abstract We used stable isotopes of C in breath, blood, feces and feathers to identify intra-individual changes in diet and the timescale of diet changes in free-living songbirds at a stopover site. Because accurate interpretation of differences between the $\delta^{13}C$ of breath, plasma, and red blood cells (RBCs) relative to diet requires knowing the turnover rate of C within them, we determined the rate of change of C in breath, plasma and RBCs for yellow-rumped warblers (*Dendroica coronata*). Half-lives of C in breath, plasma, and RBCs were 4.4 ± 2.1 h, 24.8 ± 12.3 h and 10.9 ± 3.2 days, respectively, for yellow-rumped warblers. $\delta^{13}C$ of breath, plasma, RBCs and feces from wild-caught golden-crowned kinglets (*Regulus satrapa*), ruby-crowned kinglets (*R. calendula*) and gray catbirds (*Dumetella carolinensis*) indicated that they had maintained an isotopically consistent diet for an extended period of time. However, $\delta^{13}C$ of breath and plasma indicated that white-throated sparrows (*Zonotrichia albicollis*) had recently expanded their diet to include a C_4 dietary component. Likewise, $\delta^{13}C$ of breath, plasma, RBCs and feces indicated that some wild-caught yellow-rumped warblers had consumed foods with a more enriched protein signature prior to their arrival on Block Island, and since arrival, they had consumed mostly northern bayberry (*Myrica pensylvanica*). Therefore, comparisons of the $\delta^{13}C$ of breath, plasma, RBCs, feces and feathers from individual songbirds can indicate changes in diet and provide an estimate of the timescale of the diet change.

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Introduction

Naturally occurring stable isotopes in the foods and tissues of predator and prey have been recently used to greatly improve our understanding of the nutritional ecology of free-living animals (Gannes et al. 1997, 1998; Kelly 2000; Martínez del Rio and Wolf 2004). Stable isotopes have been used to study the diets of living and extinct animals (Hilderbrand et al. 1996), to estimate trophic positions of consumers (Hobson et al. 2000a; Post 2002), and to study seasonal patterns of predation (Harding and Stevens 2001). Similarly, temporal changes in isotopic signatures have been used to study changes in diet for fish (MacAvoy et al. 2001; Post 2003), for mammals (Ben-David et al. 1997; Ayliffe et al. 2004), and for birds (Alexander et al. 1996; Haramis et al. 2001; Hobson and Bairlein 2003). Songbirds that routinely switch diets during migration (Bairlein 1987, 1990) provide a particularly interesting but largely unstudied system in which stable isotopes can be used to investigate temporal changes in the diet of free-living animals.

Few songbirds have the ability to migrate non-stop from breeding to wintering grounds and stopover sites are important to these birds for rebuilding expended energy and protein stores during migration (Moore et al. 1995; Piersma 2002). Length of stay at a stopover site is related to the birds' energetic condition upon arrival (Moore and Kerlinger 1987), and is influenced by quality of habitat at the stopover site (Russell et al. 1994; Schaub and Jenni 2001). During fall migration, especially along the east coast of the continental USA, many small songbirds change their diet from primarily insects to mostly fruits (Parrish 1997, 2000). Differences between the C isotopic signatures of tissues sampled nondestructively from the same bird may allow researchers to identify such intra-individual changes in diet, and to

identify the source of nutrients used to rebuild expended fat and protein stores in songbirds during their migration (Hobson and Clark 1993; Alexander et al. 1996; Hatch et al. 2002a).

Avian ecologists have used stable isotopes to link breeding and wintering grounds (Marra et al. 1998; Hobson 1999; Rubenstein et al. 2002), to measure nutrient allocation for reproduction (Hobson et al. 2000b), to investigate ecological links between avian species and specific prey items (Wolf and Martínez del Rio 2000; Wolf et al. 2002) and to identify avian diets (Hobson and Clark 1992a, b; Alexander et al. 1996). Despite these promising applications, there have been few stable isotope studies of small migratory birds (Kelly 2000; Hobson and Bairlein 2003; Pearson et al. 2003; Evans Ogden et al. 2004), and the lack of controlled studies of birds fed known isotopic diets makes it difficult to interpret patterns in tissue isotopic signatures observed in free-living birds (Hobson and Clark 1992b).

Our primary objective was to use stable isotopes of C in certain tissues of songbirds to identify intra-individual changes in their diet and the timescale of diet changes in free-living songbirds at a stopover site. We selected tissues that could be sampled quickly and without killing the bird, and that we expected would have significantly different turnover rates so that changes in diet over time might be detected (although breath and feces are not tissues, we use the word "tissue" to refer to them throughout this article). Hatch et al. (2002b) determined that the C in the breath of pigeons (Columba livia) had a half-life of 3.5 h, and so could be used to detect recent changes in diet. Pearson et al. (2003) estimated the half-life of C in plasma of yellow-rumped warblers (Dendroica coronata) was 9.6–16.8 h, and Hobson and Clark (1993) determined that the half-life of C in red blood cells (RBCs) of crows (Corvus brachyrhynchos) was 29.8 days. Because the rectrices of most migratory songbirds are grown on the breeding grounds (Pyle 1997), the isotopic signature of the rectrices indicates the isotopic signature of the diet during the period of feather growth (Chamberlain et al. 1997; Hobson and Wassenaar 1997; Graves et al. 2002). Thus, comparisons between the C signature of feather, breath, plasma and RBCs within an individual might indicate changes in diet of individual songbirds during fall migration. Although the aforementioned work provides estimates of half-lives for breath in pigeons, for plasma in warblers, and for RBCs in crows, no such estimates of turnover rates for all three tissues are available for small songbirds. Therefore, we estimated the rate of change for C within breath, plasma, and RBCs for yellow-rumped warblers, a common migratory songbird in North America. We then used these estimates of turnover rates to detect intra-individual changes in diet and the timescale of diet changes in five species of migratory songbirds during their stopover on Block Island, Rhode Island.

Materials and methods

Study site

All birds captured for both the field study and captive bird experiment were captured during fall migration 2001, on Block Island, Rhode Island (41°12′N, 71°35′W). Block Island is a 2,900-ha island located 19 km off the southern coast of Rhode Island. It is an important stopover site for many songbirds on their southward migration (Parrish 1997). Yellow-rumped warblers were captured throughout October and transferred to the animal care facilities on the University of Rhode Island's Kingston campus. All birds captured for laboratory work were hatch-year birds.

Determining turnover rate of breath, plasma and RBCs for yellow-rumped warblers

To determine the rate of turnover for breath, plasma and RBCs, 21 birds were randomly selected from our captive population of 65 yellow-rumped warblers. All birds were housed in stainless steel cages (51×35.5×20.5 cm) in a room with a constant light cycle (12:12 h light: dark cycle) and temperature (21°C). Birds were randomly assigned to a C_3-based ($n = 10$), or a C_4-based ($n = 11$) diet with the same macronutrient composition (25% protein, 53% carbohydrate, 12% fat), but formulated with different ingredients. The percentage of C and N concentrations of the C_4 diet were 41.4 ± 1.1 and $3.6 \pm 0.1\%$, respectively ($n = 7$), and the percentage of C and N concentrations of the C_3 diet were 46.1 ± 0.5 and $3.7 \pm 0.1\%$, respectively ($n = 7$). Percent C of the C_3 diet was slightly higher than in the C_4 diet, ($t_{12} = -10.402$; $P < 0.0005$), but the percentage of N was not different between the two diets ($t_{12} = 1.568$; $P = 0.143$). The C_3 diet was composed of beet sugar ($\delta^{13}C = -24.5\permil$), olive oil ($\delta^{13}C = -28.9\permil$) and casein ($\delta^{13}C = -26.0\permil$), and had a bulk diet signature of $\delta^{13}C = -25.2 \pm 0.2\permil$ ($n = 7$). The C_4 diet contained corn sugar ($\delta^{13}C = -9.8\permil$), corn oil ($\delta^{13}C = -15.3\permil$), and fish meal ($\delta^{13}C = -18.4\permil$), and had a bulk diet signature of $\delta^{13}C = -13.7 \pm 0.4\permil$ ($n = 7$). Both 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). Both diets were agar-based (1.25 g agar/100 g wet food) (Afik et al. 1997; Frazer and McWilliams 2002). Throughout the experiment, birds had ad libitum access to food and water, and each day they were provided with five wax-worms (Galleria mellonella) (approximately 1 g wet weight) ($\delta^{13}C = -20.8\permil$) to ensure that the long-term captive birds maintained body mass (Frazer and McWilliams 2002). Birds were weighed every day and provided with new food at 0800 hours eastern standard time. For birds scheduled to have their breath and/or

blood sampled on a given day, we delayed supplying waxworms to birds until after these tissue samples were collected.

After birds were fed these semi-synthetic diets for 2.5 months, we sampled breath and blood from six birds fed each diet (day 0). Breath samples were collected from two birds fed each diet at 1100, 1400 and 1700 hours on day 0. We collected approximately 70 µl blood from the brachial vein of six birds fed each diet at 1700 hours on day 0. We were unable to collect enough blood for isotopic analysis from one bird fed the C_4 acclimation diet. These six birds from each diet were then killed and used as part of another study. Blood samples in capillary tubes were centrifuged within 1 h of collection and the plasma and RBCs were transferred to separate cryo-vials, and then stored frozen in liquid nitrogen. The breath, plasma and RBCs from these 12 birds represent baseline values for birds fed one of these two diets for 2.5 months.

Breath samples were collected using a small facemask connected to a latex balloon as described in detail in Hatch et al. (2002a). Briefly, we flushed the balloon 5–6 times with pure oxygen and then filled the balloon with pure oxygen to just past taut. We then placed the mask over the bird's bill and face, and the excess oxygen in the balloon was released. The bird re-breathed the remaining oxygen, and the bird's exhaled CO_2 was collected in the balloon for approximately 1 min. The exhaled CO_2 was immediately transferred to an evacuated Exetainer tube (Labco, Buckinghamshire) using an 18-gauge syringe needle. Breath samples were stored at room temperature.

We then switched the diet of the remaining 11 birds from their acclimation diet to the other diet at 0800 hours on day 1. Given the expected differences in turnover rates between tissues, we sampled each tissue at different intervals of time and for a duration corresponding to 3–4 times the expected half-life. Breath samples were collected at 1100, 1400 and 1700 hours on days 1, 2, 3, and 10. Each bird was sampled once per day for breath, and the order of sampling was randomly selected. We collected approximately 70 µl blood from the brachial vein of one or two birds fed each diet at 1700 hours on days 1, 2, 3, 4, 5, 7, 9, 11, 13 and 15. We bled a given bird at most every third day to reduce possible negative effects of frequent bleeding. One bird switched from the C_3 to C_4 diet died of unknown causes on day 11 and so we had one less blood sample for birds on day 15. We ended blood sampling at 15 days because this is approximately twice the stopover duration for songbirds while on Block Island (Parrish 1997) and because we also had blood samples from birds fed each diet for > 2.5 months. Blood samples were processed as described above.

Sampling breath, plasma, RBCs, feathers and feces from birds in the field

On Block Island from 22 to 23 October 2001, between daybreak and 1500 hours, we sampled breath, plasma,

RBCs, feathers, and feces from five species of migratory songbirds including golden-crowned kinglets (*Regulus satrapa*) ($n = 10$), ruby-crowned kinglets (*R. calendula*) ($n = 2$), white-throated sparrows (*Zonotrichia albicollis*) ($n = 6$), gray catbirds (*Dumetella carolinensis*) ($n = 3$) and yellow-rumped warblers ($n = 20$). Birds were captured in mist nets, transferred to net bags and then carried to a central station. At the station, birds were weighed to the nearest 0.1 g on an electric scale and a no. 1 rectrix was removed from the bird and stored in a paper envelope. If the bird had deposited feces in the net bag, the fecal sample was placed in a glass vial and stored frozen. Breath samples were collected in the field as explained above for captive birds. We collected approximately 70 µl blood from the brachial vein of each bird. Birds were subsequently released. Within 6 h of field-collection, blood samples were centrifuged, and plasma and RBCs transferred to separate cryo-vials, and then stored frozen in liquid N.

Isotopic analysis

Plasma, RBCs, and fecal samples were freeze-dried and powdered before isotopic analysis. Feathers were cleaned prior to isotopic analysis by sonication in distilled water followed by sonication in petroleum ether. After cleaning, feathers were stored in clean paper envelopes. Scissors were used to cut a 0.10–0.25 mg sample of the feather for isotopic analysis. Plasma, RBCs, feathers, and feces 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. Samples were converted to CO_2 in oxidation/reduction furnaces and separated by gas chromatography. After separation, samples were measured for $^{13}C/^{12}C$ 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). Powdered dogfish muscle (DORM-1) reference material (National Research Council, Institute for Environmental Chemistry, Ottawa) 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.

Breath samples were analyzed at Brigham Young University using a Gasbench II on-line gas preparation and introduction system in line with a Delta Plus isotope ratio mass spectrometer (Thermo Finnigan, San Jose, Calif.). Briefly, exetainers containing the gas samples were placed in the autosampler of the Gasbench II. The sample was entrained in the carrier gas, He_2, and CO_2 was isolated through GC separation and then carried to the mass spectrometer. The external precision for CO_2 in air was approximately ± 0.2‰.

Models and statistical analysis

To quantify differences between bulk-diet signatures and tissue signatures, and differences between macronutrient signatures and tissues signatures for birds fed the acclimation diets for 2.5 months, we calculated discrimination factors (Δ_{dt}) for each tissue using the equation: $\Delta_{dt} = \delta_{(\text{tissue or macronutrient})} - \delta_{\text{diet}}$. Exponential decay curves [$Y = Y_0 + a \times \exp(-bt)$] were used to model turnover rates for C in breath, plasma and RBCs for each bird. The half-life of C in breath, plasma and RBCs was calculated using $\ln(0.5)/b$ for each bird, and averaged according to diet switch. The first data point (day 0) in each respective model was the mean $\delta^{13}C$ value of breath, plasma and RBCs collected from six birds that were sampled and then sacrificed on day 0. Also, the last data point (day 75) in each RBC model was the mean $\delta^{13}C$ value for six birds fed the same diet for 2.5 months. We used repeated-measures ANOVA (Systat) to compare the $\delta^{13}C$ values and half-life of the three tissues (breath, plasma, RBCs) for birds fed the acclimation diet for 2.5 months and for birds that had their diets switched. We used one-way ANOVA (Systat) with Bonferroni post hoc tests to compare tissue $\delta^{13}C$ values between and within free-living golden-crowned kinglets, white-throated sparrows and yellow-rumped warblers captured on Block Island. Although the use of one-way ANOVA to analyze differences in tissues from the same individual violates the assumption of independence and so involves pseudoreplication, we prefer this approach over repeated-measures ANOVA in this case because it allowed us to use all data collected from each bird and it facilitated post hoc multiple comparisons tests. Recall that our sampling protocol in the field involved opportunistically collecting feces from captured birds. Thus, use of repeated-measures ANOVA was too restrictive in this case because it required complete data for all tissues from all birds, so that 40% of birds were removed from this data set because they produced no fecal sample. We analyzed this smaller set of data using repeated-measures ANOVA and found no differences in results between the two analyses. We did not compare differences in $\delta^{13}C$ between tissues of ruby-crowned kinglets and gray catbirds because of small sample sizes. For all statistical tests, $P \leq 0.05$ was deemed significant.

Results

Estimating turnover rate for breath, plasma and RBCs

As expected, $\delta^{13}C$ of breath, plasma and RBCs from birds fed the C_3 diet was significantly more negative than that of tissues from birds fed the C_4 diet (diet treatment, $F_{3,15} = 533.591$, $P < 0.0005$) and there were significant differences between tissues within each diet treatment (tissue, $F_{2,30} = 38.606$, $P < 0.0005$; Treatment×Tissue, $F_{6,30} = 28.732$, $P < 0.0005$; Fig. 1). $\delta^{13}C$ of breath and

plasma from birds switched 10–15 days ago from the C_4 diet to the C_3 diet were similar to $\delta^{13}C$ of breath and plasma for birds fed the C_3 diet for 2.5 months. Likewise, after 10–15 days, $\delta^{13}C$ of breath and plasma from birds switched from the C_3 diet to the C_4 diet were similar to $\delta^{13}C$ of breath and plasma from birds fed the C_4 diet for 2.5 months. However, after 15 days, $\delta^{13}C$ of RBCs from birds switched from C_4 to C_3 was not similar to $\delta^{13}C$ of RBCs for birds fed the C_3 diet for 2.5 months. Likewise, $\delta^{13}C$ of RBCs from birds switched from C_3 to C_4 for 15 days was not similar to $\delta^{13}C$ of RBCs from birds fed the C_4 diet for 2.5 months (Fig. 1). Mean discrimination factors calculated using differences between bulk-diet signatures and tissue signatures, and differences between macronutrient signatures and tissue signatures are shown in Table 1.

We modeled turnover rates and calculated half-lives of C in breath, plasma and RBCs for each bird and then calculated averages across individuals fed the same diet and for all birds combined (Table 2; Figs. 2, 3). Half-life of C in breath, plasma, and RBCs was 3–6 h, 22–29 h, and 10–12 days, respectively, depending on whether the birds were switched from C_3 to C_4 or C_4 to C_3 diets (Table 2). Based on the mean half-life for all birds combined (Table 2), we estimated that 90% of the C in breath, plasma, and RBCs of a bird at any given time was incorporated within the past 12 h, 3 days, and 1 month, respectively. We fitted a single decay curve to all individual data points for each tissue for birds switched from the C_4 to C_3 diet for illustrative purposes only (Fig. 2; breath, $y = -23.05 + 9.52e^{-0.23x}$, $r^2 = 0.81$, $F_{2,18} = 39.17$,
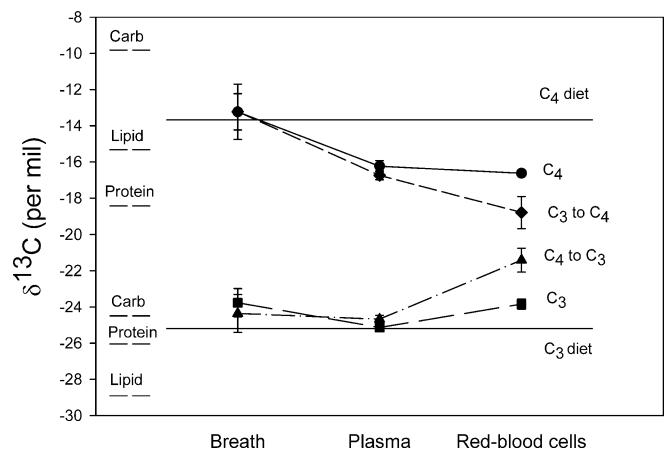

Fig. 1 $\delta^{13}C$ values (mean ± 1 SD) of breath, plasma and red blood cells (RBCs) from six birds fed acclimation diets for 2.5 months (points connected by line labeled C_3 or C_4), of breath 10 days after a diet switch [C_4 to C_3 ($n=5$) or C_3 to C_4($n=4$)], and of plasma and RBCs 15 days after a diet switch [C_4 to C_3 ($n=5$) or C_3 to C_4 ($n=3$)]. *Solid horizontal lines* represent the $\delta^{13}C$ of the C_3 and C_4 bulk diets and *dotted horizontal lines* represent the $\delta^{13}C$ of the carbohydrate (*Carb*), lipid and protein components of the two diets. Breath samples were collected at 1100, 1400, and 1700 hours each day. $\delta^{13}C$ was not significantly different between sample times within a day for birds on diets for 2.5 months (C_3, $F_{2,5} = 0.203$, $P = 0.827$; C_4, $F_{2,5} = 1.694$, $P = 0.322$), so we report the mean $\delta^{13}C$ of breath samples for a given day

Table 1 Bulk diet–tissue, carbohydrate–tissue, lipid–tissue and protein–tissue discrimination factors ($\Delta\delta^{13}C$) (mean ± 1SD) for breath, plasma and red blood cells (*RBCs*) for yellow-rumped warblers fed the C_3 and C_4 acclimation diets for 2.5 months

Diet	Tissue	Mean discrimination (‰)				
		Bulk diet	Carbohydrate	Lipid	Protein	n
C_3	Breath	1.4 ± 0.8	0.7 ± 0.8	5.1 ± 0.8	2.3 ± 0.8	6
	Plasma	0.1 ± 0.2	−0.7 ± 0.2	3.8 ± 0.2	0.9 ± 0.2	6
	RBCs	1.4 ± 0.3	0.7 ± 0.3	5.1 ± 0.3	2.2 ± 0.3	6
C_4	Breath	0.4 ± 1.0	−3.4 ± 1.0	2.1 ± 1.0	5.2 ± 1.0	6
	Plasma	−2.6 ± 0.3	−6.4 ± 0.3	−0.9 ± 0.3	2.2 ± 0.3	5
	RBCs	−3.0 ± 0.1	−6.8 ± 0.1	−1.3 ± 0.1	1.8 ± 0.1	5

Table 2 Half-life of C[a] in breath, plasma, and RBCs (mean ± 1 SD) for four birds switched from the C_3 to C_4 diet, five birds switched from C_4 to C_3, and for all birds combined

Diet switch	n	Estimate of half-life of C		
		Breath (h)	Plasma (h)	RBCs (days)
C_3 to C_4	4	5.8 ± 2.5	28.7 ± 16.3	10.0 ± 1.5
C_4 to C_3	5	3.2 ± 0.9	21.8 ± 8.7	11.7 ± 4.1
All birds	9	4.4 ± 2.1	24.8 ± 12.3	10.9 ± 3.2

[a]Half-life of C in breath, plasma, and RBCs was similar for birds switched from C_3 to C_4 diets compared to birds switched from C_4 to C_3 diets (diet treatment, $F_{1,7} = 0.363$, $P = 0.566$; diet×tissue, $F_{2,14} = 0.747$, $P = 0.492$), although half-life of C was significantly different between tissues ($F_{2,14} = 84.207$, $P < 0.0005$)

$P < 0.0001$; plasma, $y = -24.44 + 6.86e^{-0.03x}$, $r^2 = 0.92$, $F_{2,18} = 102.21$, $P < 0.0001$; RBCs, $y = -24.29 + 8.54e^{-0.07x}$, $r^2 = 0.91$, $F_{2,19} = 91.69$, $P < 0.0001$), and for birds switched from the C_3 to C_4 diet (Fig. 3; breath, $y = -13.27 - 10.90e^{-0.14x}$ $r^2 = 0.84$, $F_{2,14} = 37.89$, $P < 0.0001$; plasma, $y = -16.67 - 6.99e^{-0.16x}$, $r^2 = 0.90$, $F_{2,13} = 61.17$, $P < 0.0001$; RBCs, $y = -16.24 - 8.84e^{-0.08x}$, $r^2 = 0.93$, $F_{2,14} = 93.17$, $P < 0.0001$).

$\delta^{13}C$ of breath, plasma, RBCs, feathers and feces from free-living songbirds

White-throated sparrows had the most enriched breath $\delta^{13}C$ value compared to golden-crowned kinglets and yellow-rumped warblers ($F_{2,32} = 8.550$, $P = 0.001$) (Table 3). Yellow-rumped warblers had a more-enriched RBCs $\delta^{13}C$ value compared to golden-crowned kinglets ($F_{2,31} = 6.417$, $P = 0.005$) (Table 3). There were no significant differences between bird species in $\delta^{13}C$ of feather ($F_{2,32} = 0.510$, $P = 0.606$), feces ($F_{2,17} = 0.902$, $P = 0.424$), or plasma ($F_{2,31} = 2.167$, $P = 0.132$) (Table 3).

For all five species, feces had the most depleted isotopic signature compared to all other tissues (Table 3). For golden-crowned kinglets, gray catbirds and ruby-crowned kinglets, feather was more positive, and plasma more negative than other tissues except feces. The same pattern was apparent in yellow-rumped warblers except for their RBCs which were unusually variable (i.e., high
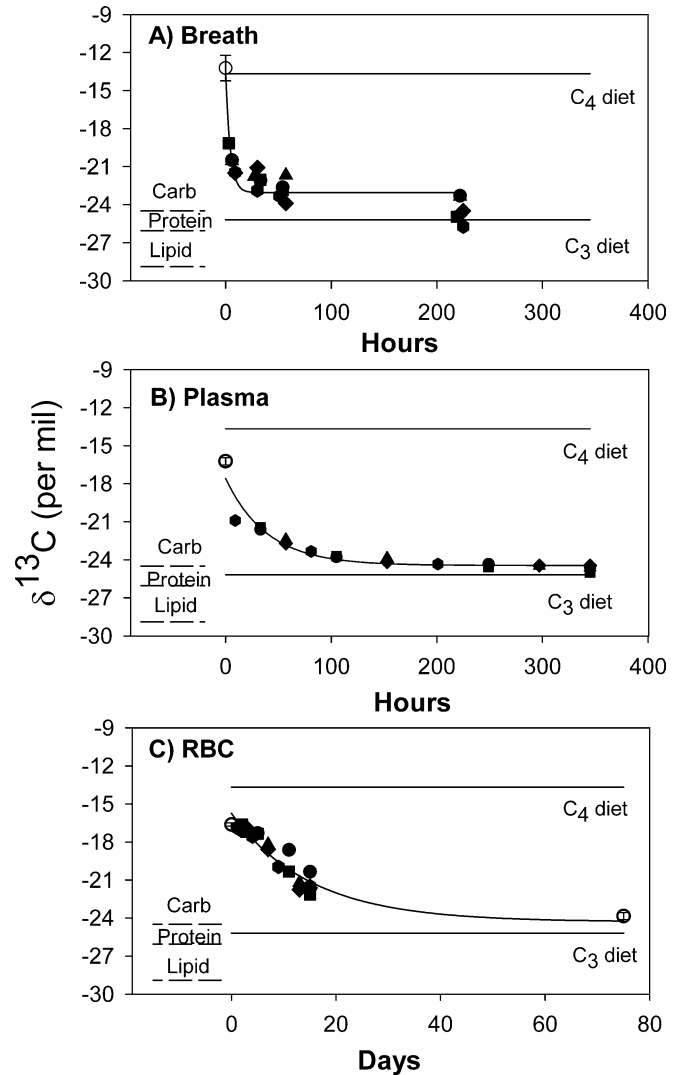

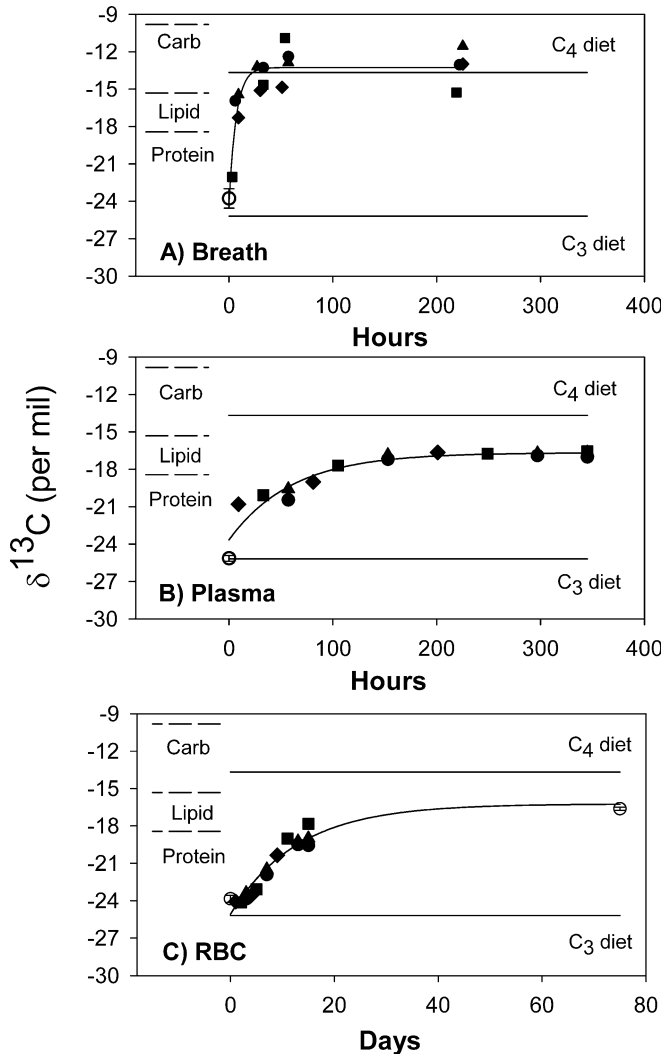**Fig. 2** Exponential decay curves for $\delta^{13}C$ in **a** breath, **b** plasma, and **c** RBCs of yellow-rumped warblers switched from a C_4 to C_3 diet. *Open circles* on day 0 are mean $\delta^{13}C$ + 1 SD of breath ($n = 6$), plasma ($n = 5$), and RBCs ($n = 5$) from birds fed the C_4 diet for 2.5 months. *Open circle* on day 75 is mean $\delta^{13}C$ + 1 SD of RBCs from six birds fed the C_3 diet for 2.5 months. Each *solid symbol* is the $\delta^{13}C$ for an individual bird (total $n = 5$ birds; see Materials and methods). *Solid horizontal lines* represent the $\delta^{13}C$ of the C_3 and C_4 diets and *dotted horizontal lines* represent the $\delta^{13}C$ signature of the carbohydrate, lipid and protein components of the C_3 diet. For abbreviations, see Fig. 1

SD), and on average more positive than other yellow-rumped warbler tissues (Table 3). All white-throated sparrow tissues except RBCs had unusually variable C signatures (i.e., high SD) compared to other tissues from the other four bird species (Table 3).

Discussion

Tissue isotope signatures for birds fed constant diets

$\delta^{13}C$ of diet strongly influenced $\delta^{13}C$ of tissues in captive yellow-rumped warblers. Tissue signatures of birds fed

Fig. 3 Exponential decay curves for $\delta^{13}C$ in **a** breath, **b** plasma, and **c** RBCs of yellow-rumped warblers switched from a C_3 to C_4 diet. *Open circles* on day 0 are mean $\delta^{13}C + 1$ SD of breath, plasma, and RBCs from six birds fed the C_3 diet for 2.5 months. *Open circle* on day 75 is mean $\delta^{13}C + 1$ SD of RBCs from five birds fed the C_4 diet for 2.5 months. Each *solid symbol* is the $\delta^{13}C$ for an individual bird (total $n = 4$ birds; see Materials and methods). *Solid horizontal lines* represent the $\delta^{13}C$ of the C_3 and C_4 diets and *dotted horizontal lines* represent the $\delta^{13}C$ signature of the carbohydrate, lipid and protein components of the C_4 diet. For abbreviations, see Fig. 1

the C_3 diet for 2.5 months were more depleted than tissue signatures of birds fed the C_4 diet for 2.5 months. Tissues from animals fed isotopically consistent diets usually reflect the signature of that diet as shown in gerbils (*Meriones unguienlatus*) (Tieszen et al. 1983), mice (*Mus musculus*) (Tieszen and Fagre 1993), and birds (Hobson and Clark 1992a; Bearhop et al. 2002). Thus, C isotope signatures can be used to identify diet of birds as long as diet signatures are distinct.

However, tissue signature is also influenced by $\delta^{13}C$ of dietary macronutrients, and by the composition of the tissue (Pearson et al. 2003; Martínez del Rio and Wolf 2004). Our data indicate that macronutrient routing and tissue composition also influenced $\delta^{13}C$ of warbler tis-

sues. For example, bulk diet-tissue discrimination factors for $\delta^{13}C$ ($\Delta\delta^{13}C$) ranged from $0.1 \pm 0.2\permil$ for plasma to $1.4 \pm 0.8\permil$ for breath of birds fed the C_3 diet, and from $-3.0 \pm 0.1\permil$ for RBCs to $0.4 \pm 1.0\permil$ for breath of birds fed the C_4 diet (Table 1). The range in discrimination factors for birds fed the C_3 diet is within the range found by other researchers, however, the range in discrimination factors for birds fed the C_4 diet is generally more negative than found by other researchers (DeNiro and Epstein 1978; Tieszen et al. 1983; Mizutani et al. 1992; Hobson and Bairlein 2003; Pearson et al. 2003).

If dietary protein is preferentially routed to proteinaceous tissues (Martínez del Rio and Wolf 2004), then discrimination factors for plasma and RBCs may be more accurately calculated using dietary protein signature rather than bulk-diet signature. Discrimination factors calculated between $\delta^{13}C$ of dietary protein and $\delta^{13}C$ of plasma and RBCs for birds fed the C_3 diet were 0.9 ± 0.2 and $2.2 \pm 0.3\permil$ respectively, and dietary protein discrimination factors for birds fed the C_4 diet were 2.2 ± 0.3 and $1.8 \pm 0.1\permil$ respectively (Table 1), which are within the range found by other researchers for bulk diet (DeNiro and Epstein 1978; Hobson and Clark 1992a; Mizutani et al. 1992). The influence of dietary protein on discrimination factors for plasma and RBCs may have been more evident for birds fed the C_4 diet than birds fed the C_3 diet because the difference between the $\delta^{13}C$ of the dietary protein in the C_4 diet and the $\delta^{13}C$ of the bulk C_4 diet ($4.8\permil$) was greater than the difference between the $\delta^{13}C$ of the dietary protein in the C_3 diet and the $\delta^{13}C$ of the bulk C_3 diet ($0.9\permil$).

Assuming that dietary protein is preferentially routed to proteinaceous tissues such as RBCs, $\delta^{13}C$ of breath should reflect the composition of the macronutrients that are catabolized for energy after protein requirements are satisfied (Martínez del Rio and Wolf 2004). The larger difference between $\delta^{13}C$ values of macronutrients within the C_4 diet, combined with the highly enriched signature of the carbohydrate source ($\delta^{13}C = -9.8\permil$) in the C_4 diet could explain why breath was more enriched than RBCs and plasma for birds fed the C_4 diet for 2.5 months (Fig. 1). In summary, our results demonstrated that tissue $\delta^{13}C$ was influenced by the $\delta^{13}C$ of bulk diet, by the $\delta^{13}C$ of macronutrients within the diet, and by the nutrient composition of the tissue.

Detecting diet switching of individuals by comparing isotopic signatures of their tissues

To reliably identify intra-individual changes in diet for small songbirds using isotope signatures of tissues, the rate of isotopic change between different tissues must be different. We estimated half-life of C in breath to be approximately 3–6 h depending on diet. Hatch et al. (2002b) estimated a half-life of 3.5 h for breath C in pigeons. We estimated half-life of C in plasma to be

Table 3 $\delta^{13}C$ values (mean $\pm$ 1 SD) of feather, breath, RBCs, plasma and feces from golden-crowned kinglets (*GCKI*), gray catbirds (*GRCA*), ruby-crowned kinglets (*RCKI*), white-throated sparrows (*WTSP*), and yellow-rumped warblers (*YRWA*) captured on Block Island, Rhode Island, during fall migration in October 2001. GRCA and RCKI were excluded from statistical analysis because of small sample size. $\delta^{13}C$ values in the same row with the *same letter* are not significantly different ($P > 0.05$). $\delta^{13}C$ values for breath and RBCs *in italics* are not significantly different ($P > 0.05$)

Species	Feather	Breath	RBC	Plasma	Feces	F-value[a]	P-value[a]
GRCA	-22.9 ± 0.3	-24.1 ± 0.7	-25.2 ± 0.2	-26.0 ± 0.4	-28.9 ± 0.6		
	$n = 3$	$n = 3$	$n = 3$	$n = 3$	$n = 2$		
RCKI	-22.5 ± 0.0	-24.5 ± 0.5	-22.8 ± 1.1	-24.8 ± 0.9	-26.6 ± 0.4		
	$n = 2$	$n = 2$	$n = 2$	$n = 2$	$n = 2$		
WTSP	-23.6 ± 2.6 ab	-21.2 ± 3.1 a	*-22.8 ± 0.3* ab	-23.5 ± 2.6 ab	-26.3 ± 2.4 b	2.973	0.0420
	$n = 6$	$n = 6$	$n = 5$	$n = 5$	$n = 5$		
YRWA	-23.1 ± 1.0 a	*-23.9 ± 0.8* ab	-21.5 ± 2.6 c	-24.6 ± 1.2 b	-27.2 ± 0.7 d	28.533	≤ 0.0001
	$n = 20$	$n = 20$	$n = 19$	$n = 20$	$n = 11$		
GCKI	-22.9 ± 0.6 a	*-23.4 ± 0.6* ab	-24.2 ± 0.3 b	-25.1 ± 0.8 c	-26.4 ± 0.6 c	35.006	≤ 0.0001
	$n = 9$	$n = 9$	$n = 10$	$n = 9$	$n = 4$		

[a]One-way ANOVA comparing tissue $\delta^{13}C$ for WTSP, YRWA, and GCKI; numerator $df = 4$ for all species; denominator $df = 36$ for GCKI, 22 for WTSP, and 85 for YRWA

approximately 25 h. Pearson et al. (2003) estimated half-life of C in plasma to be 9.6–16.8 h for yellow-rumped warblers. The half-life we calculated for plasma C was likely longer because we did not sample all birds on day 1. Half-life of plasma from crows, which are 38 times larger than warblers, was 2.9 days (Hobson and Clark 1993). These differences between studies suggest that diet and body size of birds influence plasma turnover rate (Hobson and Bairlein 2003). However, if a small songbird changes its diet upon arrival at a stopover site, our results, along with those of Pearson et al. (2003), suggest that plasma can reflect the new diet within 1–2 days of feeding.

As expected, C in RBCs had the longest turnover of the tissues sampled. We calculated a half-life for RBCs of approximately 11 days, which is much faster than that reported for crows (29.8 days) (Hobson and Clark 1993). These results suggest that smaller birds such as warblers have faster C turnover rates than larger birds (Hobson and Bairlein 2003), which clearly has implications for accurately estimating the timing of diet switching in a given species. Because the turnover of RBCs is longer than plasma or breath, yet still within the overall time a small songbird spends migrating, measuring $\delta^{13}C$ of breath, plasma, RBCs and feathers allows the detection of diet changes of migrant songbirds that occurred hours, days or weeks prior to capture.

Interpreting the isotopic signatures of tissues sampled from free-living songbirds

Our captive studies demonstrated how diet switching quantitatively affects isotopic signatures of certain tissues, and allowed us to directly measure turnover rates of these tissues in small songbirds. Based on this captive work, if a bird ate a diet for an extended period of time that was as homogeneous as our C_3 diet, then we expect the $\delta^{13}C$ of breath, plasma and RBCs to be < 1–2‰ enriched compared to the $\delta^{13}C$ of bulk diet. However, if a bird ate a more heterogeneous diet (e.g., our C_4 diet) for an extended period of time, then we expect the $\delta^{13}C$ of these tissues to relate less to the $\delta^{13}C$ of bulk diet and more to the $\delta^{13}C$ of the macronutrient(s) primarily composing the tissue. Thus, $\delta^{13}C$ of breath, plasma and RBCs are expected to be quite different (i.e., > 2‰) when a bird eats a heterogeneous diet composed of macronutrients with distinct $\delta^{13}C$ signatures. However, regardless of the uniformity of the isotopic signatures of the macronutrients within a diet, if all birds within a population ate the same diet, we expect the $\delta^{13}C$ value of a given tissue to be consistent across individuals (i.e., low SD).

In contrast, if a bird switched diets within the past month and these diets are isotopically distinct, then we expect the $\delta^{13}C$ of breath, plasma and RBCs to be quite different (i.e., > 2‰). Evidence of a recent change in diet can be strengthened if $\delta^{13}C$ of feather provides an estimate of the $\delta^{13}C$ of a bird's past diet and it differs appreciably from the $\delta^{13}C$ of its current diet, as indicated by $\delta^{13}C$ of tissues with rapid turnover (e.g., breath, plasma). The timing of this recent change in diet can be inferred by identifying which tissue(s) $\delta^{13}C$ values are most different and applying our estimates of tissue half-life. We now use this information to identify changes in diet for wild-caught songbirds.

$\delta^{13}C$ of breath, plasma and RBCs from free-living golden-crowned kinglets, gray catbirds and ruby-crowned kinglets were < 2‰ different across tissues for each species, and were similar in pattern to $\delta^{13}C$ of breath, plasma and RBCs from captive yellow-rumped warblers fed the C_3 diet for 2.5 months. In addition, the intra-tissue variation in $\delta^{13}C$ signatures within each species was small (i.e., SD < 1.1‰), and the $\delta^{13}C$ of feathers for the three species had C_3 signatures (Table 3). These patterns in isotopic signatures of tissues suggest that these three species had eaten food with a relatively consistent C_3 signature for an extended period of time.

$\delta^{13}C$ of breath, plasma and RBCs from free-living white-throated sparrows were > 2‰ different, and $\delta^{13}C$

of breath and plasma were heterogeneous across the population (i.e., high SD), whereas $\delta^{13}C$ of RBCs was homogeneous across the population (i.e., low SD) (Table 3). This high variation in $\delta^{13}C$ of breath and plasma was caused in part because one white-throated sparrow had unusually enriched breath ($\delta^{13}C = -15.4\%_o$) and plasma ($\delta^{13}C = -19.4\%_o$) signatures. We also collected a rectrix from this bird that was emerging from its sheath. $\delta^{13}C$ of this rectrix ($\delta^{13}C = -18.6\%_o$) was also highly enriched. These isotopic patterns suggest that the white-throated sparrow with the enriched breath, plasma and feather signatures had recently expanded its diet to include food with a strong C_4 signature (e.g., corn found in birdfeeders). Interestingly, the other white-throated sparrows also appeared to have recently expanded their diet to include the C_4 diet component. $\delta^{13}C$ of breath, plasma and RBCs from these other white-throated sparrows were $> 2\%_o$ different, and $\delta^{13}C$ of breath was more enriched than for the other wild species sampled. Thus, in contrast to the other bird species mentioned above, all white-throated sparrows we sampled appear to have recently made a significant change in their diets within 3 days of capture.

$\delta^{13}C$ of breath, plasma and RBCs from free-living yellow-rumped warblers were $> 2\%_o$ different, and $\delta^{13}C$ of RBCs was heterogeneous across the population (i.e., high SD; range from -18.0 to $-24.5\%_o$); whereas $\delta^{13}C$ of breath and plasma were homogeneous across the population (i.e., low SD) (Table 3). If yellow-rumped warblers spend, on average, 5–6 days on Block Island during migration (Parrish 1997), then these results suggest that some yellow-rumped warblers had changed their diet before arriving on Block Island. To better illustrate which yellow-rumped warblers had switched their diets before arriving on Block Island, we plotted $\delta^{13}C$ values for feather, RBCs, plasma, breath and feces collected from all wild yellow-rumped warblers. As can

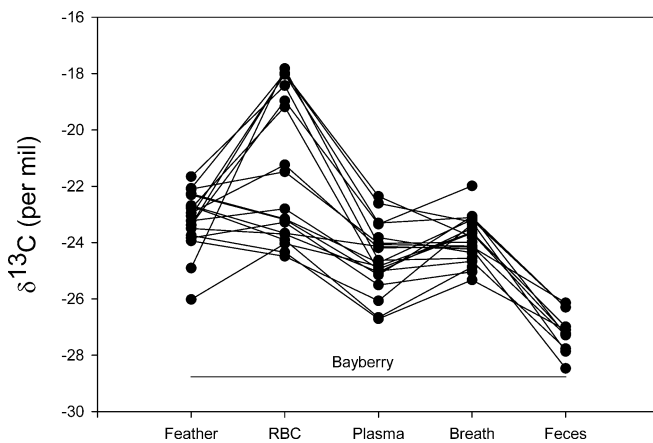**Fig. 4** $\delta^{13}C$ values of the feathers, RBCs, plasma, breath and feces for individual wild-caught yellow-rumped warblers ($n = 20$) sampled on Block Island during fall 2001. Symbols connected by *lines* represent data from the same individual yellow-rumped warbler. *Solid horizontal line* represents $\delta^{13}C$ of northern bayberry (*Myrica pensylvanica*)

be seen from Fig. 4, a group of yellow-rumped warblers had RBCs with distinctly positive $\delta^{13}C$ values. We expected the feathers of these birds to have $\delta^{13}C$ values between -22.0 and $-25.0\%_o$ because the rectrices were grown on or near the breeding grounds (Chamberlain et al. 1997; Rubenstein et al. 2002). Given that yellow-rumped warblers during migration consume mostly northern bayberry (*Myrica pensylvanica*) on Block Island (Parrish 1997, 2000) and elsewhere (Place and Stiles 1992), we hypothesized that C signatures of breath, plasma, RBCs, and feces would be influenced by the C signature of northern bayberry ($\delta^{13}C = -28.6\%_o$), but to different extents, depending on the timing of the change to northern bayberry and the turnover rate of the tissues.

As expected, wild-caught yellow-rumped warblers had feather $\delta^{13}C$ signatures ranging from -21.7 to $-26.0\%_o$, and had consumed large amounts of northern bayberry while on Block Island, as indicated by the presence of northern bayberry seeds in all fecal samples. In addition, for most yellow-rumped warblers, $\delta^{13}C$ of breath, plasma and feces were between the $\delta^{13}C$ signature of their feathers and $\delta^{13}C$ of northern bayberry. However, $\delta^{13}C$ of RBCs from some wild-caught yellow-rumped warblers was highly enriched relative to feather $\delta^{13}C$. These results suggest that prior to arrival on Block Island some of the wild-caught yellow-rumped warblers had changed their diet from the diet they had been eating when their feathers were grown. Assuming that our estimates of C turnover from captive yellow-rumped warblers apply to field-caught birds, the group of wild-caught yellow-rumped warblers that switched their diets had consumed a diet with a more positive signature for approximately 2–3 weeks prior to arrival on Block Island, and since arrival on Block Island, had begun to consume large amounts of northern bayberry as demonstrated by the $\delta^{13}C$ signature of their breath, plasma and feces.

Determining the specific diets consumed by these warblers prior to their arrival on Block Island is difficult because the observed pattern of $\delta^{13}C$ across tissues could be produced by more than one type of diet. Recall that feathers and RBCs are mostly protein and so the $\delta^{13}C$ of these tissues is most similar to that of dietary protein. Thus, the previous diet that caused the enriched $\delta^{13}C$ of RBCs in some warblers had a more positive $\delta^{13}C$ of its dietary protein, but the $\delta^{13}C$ of the other macronutrients in the diet could be similar to or quite different than that of dietary protein. We may often not know for certain the actual diet of a migratory bird at its previous stop-over site given only tissue isotope signatures; however, certain patterns in the $\delta^{13}C$ of breath, plasma, RBCs and feathers of a bird indicate that this individual has eaten isotopically distinct diets.

Assumptions and caveats

The efficacy of using patterns of C signatures in breath, blood, feathers, and feces to detect intra-individual

changes in diet requires: (1) that the diets of interest are isotopically distinct, (2) that the diet switch occurred while the C in at least one of these tissues was being incorporated, and (3) that factors known to produce differences in C signature between diet and bird tissue, including dietary C and N concentration (Pearson et al. 2003), and metabolic routing of dietary nutrients and fractionation (Martínez del Rio and Wolf 2004), produce changes in C isotope signatures of tissues that are relatively small compared to the changes due to the diet switch. For our captive study, we formulated each diet to be isotopically distinct. However, recall that macronutrient signatures of the C_4 diet differed more than that of the C_3 diet and this resulted in birds fed the C_4 diet having greater differences in C signature between tissues compared to birds fed the C_3 diet. These effects of fractionation and metabolic routing of macronutrients into tissues that differ in composition can obscure evidence of diet switching derived from intra-individual comparisons of C signatures in bird tissues. For example, we detected no diet switching in three of the five species of wild birds that we studied although it is possible that these three species were switching between isotopically similar diets. However, for yellow-rumped warblers and white-throated sparrows in our field study, the patterns of C signature in bird tissues were consistent with the hypothesis that these free-living birds had recently changed their diet while on migration. As we understand more about the isotope signatures of constituents of natural foods of wild birds, and how certain physiological processes produce differences in isotope signature between consumers and their food, it will become clearer where and when we can use stable isotopes of tissues to detect diet switching in free-living birds.

Conclusions

We combined direct measures of C turnover rates in non-destructively sampled tissues of small songbirds with collections of these same tissues from wild birds to identify changes in diet of wild birds and the time scale of the diet changes. Ornithologists can combine these stable isotope techniques with more traditional techniques such as gut-content analysis, fecal analysis and observation to determine temporal patterns of diet choice in birds. Given that birds need to rebuild both energy and protein stores catabolized during migration (Bauchinger and Biebach 1998; Bordel and Haase 2000; Piersma 2002), stable isotope techniques are especially useful for identifying resources used by birds to replenish their energy and nutrient stores. For example, if fruits supply adequate carbohydrates and fat for energy but have inadequate amounts of protein for rebuilding and repairing proteinaceous tissue (Levey and Karasov 1989; Witmer 1998), then stable isotope signatures of tissue protein in birds may identify the dietary source of protein (Martínez del Rio and Wolf 2004). This suggests that analysis of the signatures of macronutrients within

the diet of wild birds would help explain how birds assimilate specific dietary nutrients. Consequently, experiments that manipulate the proportion and isotopic signatures of the macronutrients within the diet are needed to elucidate the mechanisms that animals use to fatten and repair tissue on varying and changing diets.

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