Dynamics of Individual Fatty Acids in Muscle Fat Stores and Membranes of a Songbird and Its Functional and Ecological Importance

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Accepted 11/28/2018; Electronically Published 2/11/2019

Online enhancements: supplemental table and figures.

ABSTRACT

Although tissue fatty acid (FA) composition has been linked to whole-animal performance (e.g., aerobic endurance, metabolic rate, postexercise recovery) in a wide range of animal taxa, we do not adequately understand the pace of changes in FA composition and its implications for the ecology of animals. Therefore, we used a C_4 to C_3 diet shift experiment and compound-specific $\delta^{13}C$ analysis to estimate the turnover rates of FAs in the polar and neutral fractions of flight muscle lipids (corresponding to membranes and lipid droplets) of exercised and sedentary zebra finches (*Taeniopygia guttata*). Turnover was fastest for linoleic acid (LA; 18:2n6) and palmitic acid (PA; 16:0), with 95% replacement times of 10.8–17.7 d in the polar fraction and 17.2–32.8 d in the neutral fraction, but was unexpectedly slow for the long-chain polyunsaturated FAs (LC-PUFAs) arachidonic acid (20:4n6) and docosahexaenoic acid (22:6n3) in the polar fraction, with 95% replacement in 64.9–136.5 d. Polar fraction LA and PA turnover was significantly faster in exercised birds (95% replacement in 8.5–13.3 d). Our results suggest that FA turnover in intramuscular lipid droplets is related to FA tissue concentrations and that turnover does not change in response to exercise. In contrast, we found that muscle membrane FA turnover is likely driven by a combination of selective LC-PUFA retention and consumption of shorter-chain FAs in energy metabolism. The unexpectedly fast turnover of membrane-associated FAs in muscle suggests that songbirds during migration could substantially remodel their membranes within a single migration stopover, and this may have substantial implications for how the FA composition of diet affects energy metabolism of birds during migration.

Keywords: fatty acids, lipid droplet, membrane, metabolic rate, songbirds, turnover, compound-specific stable isotope analysis.

Introduction

Fatty acid (FA) composition of animal tissues is related to ecologically relevant measures of whole-animal performance, such as aerobic endurance, metabolic rate, postexercise recovery periods, and overwintering behavior, in a diversity of animals, including songbirds (Pierce et al. 2005; McWilliams and Pierce 2006; Price and Guglielmo 2009), salmon (McKenzie and Higgs 1998), rodents (Ayre and Hulbert 1997; Diedrich et al. 2014), humans (Lenn et al. 2002; Mickleborough et al. 2006), and zooplankton (Mariash et al. 2017). These relationships occur because the chemical properties of FAs strongly influence their function in biological systems, shaping contributions to cell structure, intercellular signaling, gene regulation, and energy storage. For example, membrane fluidity and permeability (Stubbs and Smith 1984), as well as the activity of some membrane-bound enzymes (Maillet and Weber 2007; Arnold et al. 2015), both depend on the FA composition of cell and organelle membranes. Similarly, the energy density and biochemical availability of intracellular energy stores depend on the FA composition of lipid droplets (Raclot 2003; Price et al. 2008; Guglielmo 2010). The susceptibility to oxidative damage (Hulbert 2010; Skrip and McWilliams 2016) and signaling use (Watkins 1991; Sampath and Ntambi 2004) of all FAs depends on the number and placement of double bonds in their acyl chains. Thus, understanding the determinants of FA composition of animal tissues and its time course are key steps toward predicting the function and fitness of individual animals in ecological contexts.

Although the FA composition of membranes and lipid stores plays an important role in physiological function, it can be quite dynamic in response to both endogenous and exogenous factors (Blem 1976; McWilliams et al. 2004), with the turnover of molecules in tissues being an essential step in compositional changes (Zollitsch et al. 1997; Sanz et al. 2000; McCue et al. 2009). Endogenous influences include variation in the activity of lipogenic enzymes that modify existing FAs (Egeler et al. 2000; Shimozuru et al. 2012) and the selective oxidation and storage of tissue and dietary FAs (Raclot 2003; Pierce and McWilliams 2005; Price et al. 2008, 2010). The primary exogenous influence is dietary

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Physiological and Biochemical Zoology 92(2):239–251. 2019. 1522-2152/2019/9202-8068$15.00. DOI: 10.1086/702667

availability of FAs (Ayre and Hulbert 1996; Pierce et al. 2004; McCue et al. 2009), which can vary both across individuals' diets (Frank et al. 2008) and within an individual's diet (Bairlein 1998; Smith et al. 2007; Klaiman et al. 2009). Animals also exclusively rely on diet as a source of ω-3 and ω-6 polyunsaturated FAs (PUFAs), which cannot be synthesized by vertebrates (Klasing 1998; Stevens 2004). The direct routing of these FAs from diet to consumer is particularly important because PUFAs appear to drive most lipid-based functional changes in whole-animal performance (Nagahuedi et al. 2009; Price 2010; Pierce and McWilliams 2014; Arnold et al. 2015).

Despite the dynamic nature of FA composition in animal tissues, we do not yet adequately understand the pace of turnover for individual FAs and consequently the pace of changes in tissue FA composition and their subsequent implications for animal performance. Without this knowledge, it is unclear whether changes in performance happen on ecologically relevant timescales, and our ability to estimate future performance is severely limited. Additionally, rates of isotopic incorporation are essential for the successful use of stable isotope analysis in evaluating animal diets, species interactions, and even ecophysiological changes in response to energetic challenges, such as migration or fasting (Wolf et al. 2009; Bauchinger and McWilliams 2010; Ben-David and Flaherty 2012; Martínez del Rio and Carleton 2012). With the increasing analysis of the isotopic composition of individual amino acids and FAs (Evershed et al. 2008; Graham et al. 2014; Gómez et al. 2018; Nielsen et al. 2018), the need for compound-specific turnover data is becoming more urgent, particularly for FAs. These compound-specific turnover rates may differ among species and tissues but for FAs are also likely to differ among membranes and intracellular lipid droplets, given the substantial differences in bulk turnover between those fractions (Carter et al. 2018). In general, turnover may be influenced by diet composition, tissue composition, and catabolism (either functional [e.g., energy metabolism] or nonfunctional [e.g., oxidative damage]; fig. 1). Changes in each of these variables could produce variation in turnover among lipid fractions. Thus, evaluating the relative importance of these mechanisms is necessary to explain broad trends in turnover and more completely understand dynamic changes in FA composition.

Understanding these dynamics is especially important in songbirds, whose dietary FA composition has been directly linked to their tissue FA composition, growth, and metabolic rate (Price 2010; Pierce and McWilliams 2014; Martínez del Rio and McWilliams 2016; Twining et al. 2016*b*). Moreover, the high energetic demands of powered flight and the reliance of birds on fat as fuel (Butler and Woakes 1990) increase the sensitivity of songbirds to FA-driven changes in performance (McClelland 2004; Guglielmo 2010; Pierce and McWilliams 2014), and many songbird species seasonally shift from an insect-based to a fruit-based diet (Parrish 1997; Smith and McWilliams 2014), which changes the FA profile of their diets. Here we investigate the dynamics of FA composition in the flight muscle of a model songbird, the zebra finch (*Taeniopygia guttata*), by measuring the turnover rates of individual FAs in polar and neutral lipid fractions, which correspond to muscle membranes and lipid stores, respectively. With stable body conditions and FA compositions, we expected turnover to be faster for PUFAs than for monounsaturated (MUFA) or saturated (SFA) FAs in both neutral and polar fractions due to their higher susceptibility to oxidative damage and more rapid mobilization. We also imposed a flight training regime on a subset of birds to determine whether the elevated energy demands of exercise increased FA turnover. We also calculated carbon isotope discrimination factors ($\Delta^{13}C$), which are also necessary for isotope-based diet reconstructions (Budge et al. 2008; Graham et al. 2014). To our knowledge, this is the first study to measure the turnover of individual FAs in the tissues of adult birds and the first to test the effect of elevated metabolic rate from exercise on the dynamics of FA composition.

Methods

Housing, Diets, and Experimental Treatments

We housed 65 adult zebra finches in four single-sex aviaries (2.2 m × 1 m × 2 m) with a 12L:12D light schedule and an air temperature of 23°–27°C. Throughout the experiment, birds had access to ad lib. food, water, mineral-enriched grit, and cuttlebone, except during the periods of daily flight training described below. During the ~3-mo equilibration phase of the experiment (days −90 to 0), we fed birds a C_4 mixed seed diet with a bulk $\delta^{13}C$ of −15.6‰ ± 0.3‰ (Hagen B2405, Mansfield, MA) primarily composed of millet (*Pennisetum glaucum*; table S1, available online). On day 0, we switched birds to a C_3 mixed seed diet with the much lower bulk $\delta^{13}C$ of −27.7‰ ± 0.1‰ (Abbaseed 3700, Hillside, NJ) primarily composed of canary grass (*Phalaris canariensis*; Abdel-Aal et al. 1997; table S1) for the remainder of the experiment (until day 256). To monitor bird health, we measured the fat score (0 [no visible fat] to 7 [complete fat coverage]; adapted from Eck et al. 2011) and body mass of each individual weekly.

At the start of the experiment, birds were randomly sorted into two experimental groups: control ($N = 32$, 15 males, 17 females) and exercised ($N = 33$, 16 males, 17 females). The exercised group received 2 h of flight training daily for 10 wk, from day −14 until day 56. During flight training, a handler prompted birds to fly as a flock clockwise around a 6 × 3 × 2-m flight arena between perches in opposite corners. This resulted in approximately 8.4 km of short-burst flights per day, which is ~3× more energetically costly than sustained flight for small songbirds (Nudds and Bryant 2000). To match the fasting of exercised birds during flight training, we removed food during the same 2-h exercise period each day from birds in the control group, which remained in their aviaries throughout.

Tissue Sampling and Lipid Extraction

We euthanized two to four birds from each experimental group (one or two birds of each sex) on days 0, 1, 2, 4, 8, 16, 33, 56, 120, and 256 after the diet shift. On a given day, all birds to be euthanized were placed in cloth bags immediately after flight training and held for less than 80 min before euthanization. Birds were weighed to the nearest 0.1 g, checked for fat score, and

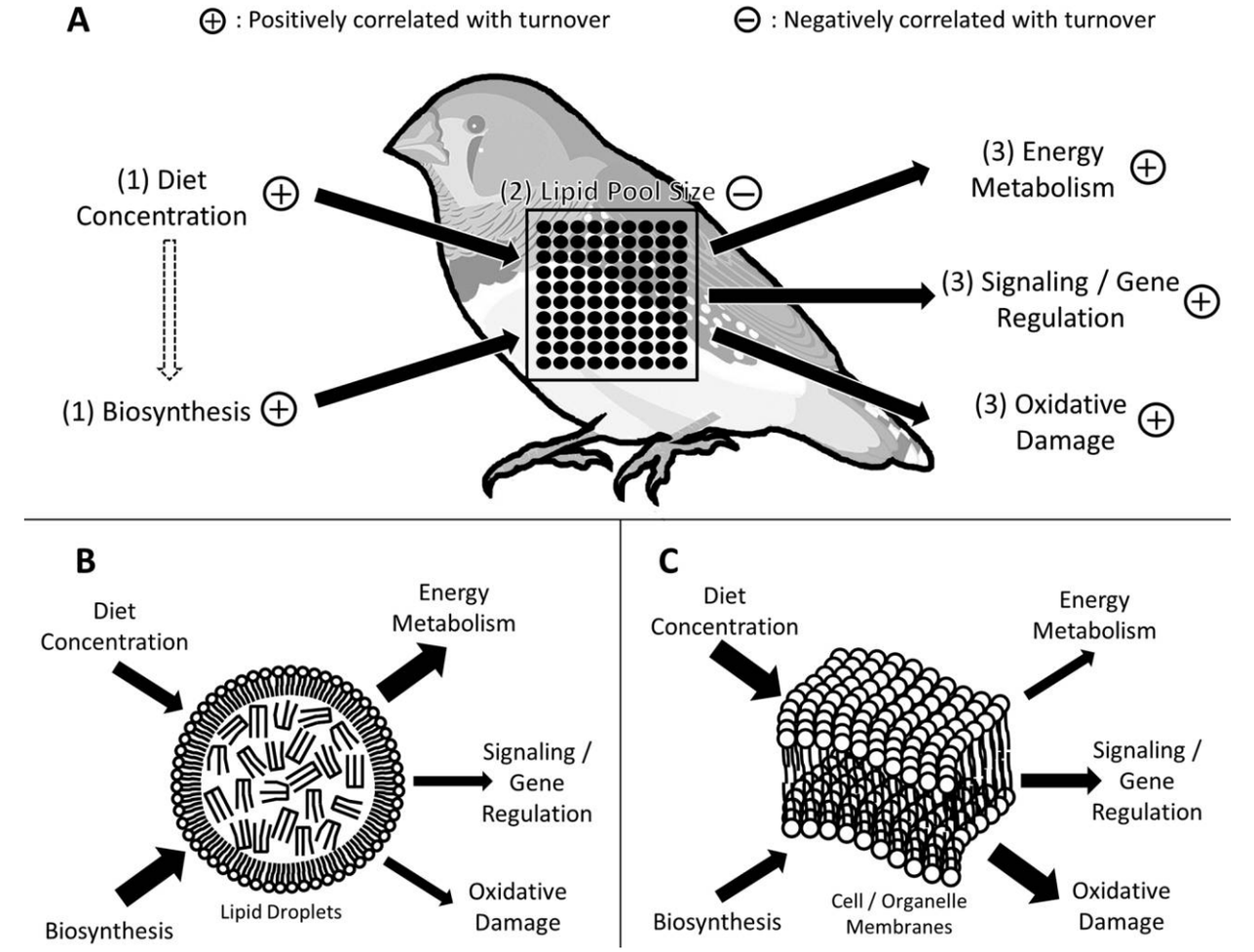

Figure 1. Factors hypothesized to influence fatty acid turnover in animal tissues. *A*: 1, High dietary concentrations or synthesis of a fatty acid coincident with no change in pool size may increase the dilution of molecules already present in the tissue, resulting in positive correlations with turnover rates. 2, Larger tissue pools take longer to dilute or to be catabolized, resulting in a negative correlation between pool size and turnover rate. 3, Higher rates of catabolism via energy metabolism, the use of fatty acids as hormones and gene regulator ligands, or oxidative damage may remove fatty acids more quickly from animal tissues, resulting in positive correlations with turnover rates. The relative strength of these factors (arrow size) is expected to differ between neutral and polar fractions of fatty acids; anatomically, these fractions correspond to lipid droplets (*B*) and cell and organelle membranes (*C*), respectively. Fatty acids in lipid droplets tend to be more readily synthesized saturated (SFA) and monounsaturated (MUFA) molecules and are generally considered to be energy stores for fueling metabolism. Cell and organelle membranes tend to have higher concentrations of less-readily synthesized polyunsaturated fatty acids (PUFAs), which are also more commonly associated with hormonal and gene regulation functions, leading to potentially greater relative importance of diet composition and signaling. PUFAs are also more susceptible to oxidative damage than SFAs or MUFAs, which is compounded by the elevated exposure of fatty acids in mitochondrial membranes to reactive species.

decapitated, and the pectoralis muscles and other selected organs were removed within 10 min. Tissue samples were then rinsed in water, blotted dry, weighed to the nearest 0.1 mg, flash frozen in liquid nitrogen, and stored at −80°C until further analysis. Sampling on day 0 immediately preceded the diet shift, so those birds had been fed only the C_4-based diet. We concentrated sampling in the days immediately following the diet shift to capture the period of expected greatest change in $\delta^{13}C$ values. Samples on days 120 and 256 were included to allow robust fitting of exponential decay models (see below; Bauchinger and McWilliams 2009, 2010). All euthanization and tissue-sampling procedures were approved by the University of Rhode Island Institutional Animal Care and Use Committee under protocol AN-12-009.

We extracted total lipids from pectoralis samples using a modified Folch method (Folch et al. 1957; Guglielmo et al. 2002). Approximately 400 mg of wet tissue was homogenized in 6 mL of 2 : 1 chloroform to methanol with a high-speed stainless steel homogenizer (PowerGen 700; Fisher Scientific, Waltham, MA) and centrifuged at 3,000 rpm for 15 min, and then aqueous solutes were separated by rinsing with 0.25% KCl. The organic phase was transferred to a 4-mL glass vial by Pasteur pipette, dried under N_2, and resuspended in chloroform. Neutral lipid, nonesterified

fatty acid (NEFA), and polar fractions were separated in solid phase extraction columns (Supelco, LC-NH_2, 1 mL aminopropyl bonding) with sequential elusions of 2:1 chloroform to isopropanol, 49:1 isopropyl ether to acetic acid, and methanol. Neutral and polar fractions were collected in 4-mL vials while the NEFA fraction was discarded. Fractionated samples were then esterified into FA methyl esters (FAMEs) by heating at 90°C for 2 h in 1M acetyl chloride in methanol.

FA Analyses

We determined FA concentrations in a subset of our samples from each fraction ($N = 51$; neutral = 26, polar = 25) using gas chromatography–flame ionization (GC-FID). Duplicate 1-μL aliquots of sample FAMEs (1 mg/mL in dichloromethane) were injected into a Shimadzu Scientific Instruments QP2010S GC-MS linked to a 2010 FID (Shimadzu Scientific Instruments) at Sacred Heart University (Fairfield, CT). Peaks were identified by retention times established by analysis of GLC standard FAME mixes (Nu-Chek Prep, Elysian, MN) run every 15 samples and visual inspection of all chromatograms. Concentrations of individual FAs were calculated as a percent by mass (FA peak area/total chromatogram area).

We quantified $\delta^{13}C$ of individual FAs in our samples and experimental diets with a Trace 1310 GC linked to a Delta V Plus isotope ratio mass spectrometer via an Isolink II combustion oven and Conflo IV reference gas interface (all instruments made by Thermo Scientific) at the University of New Mexico Center for Stable Isotopes (Albuquerque, NM). Sample FAMEs were suspended in hexane at a concentration of 0.12 mg/mL for neutral fraction samples and 0.20 mg/mL for polar fraction samples, and a 1-μL aliquot was injected into the GC (splitless mode). Samples were run in duplicate, and all chromatograms were individually inspected. After every third sample, we injected a 16:0 standard (Nu-Chek Prep) of known $\delta^{13}C$ that had been methylated alongside unknown samples in the same batch and used the offset between measured and known $\delta^{13}C$ values for this standard to correct sample $\delta^{13}C$ values for the addition of a methyl group during esterification. Carbon isotope values are presented in delta (δ) notation relative to Vienna Pee Dee Belemnite (VPDB): $\delta^{13}C‰ = [(R_{sample}/R_{standard}) - 1] \times 1{,}000$, where $R = {}^{13}C/{}^{12}C$ of the sample and the standard, respectively. Analytical precision, estimated as the within-run standard deviation of our 16:0 standard, was 0.3‰.

Data Analysis

All statistical analyses were conducted in R version 3.2.5 (R Core Team, Vienna). Preliminary data analyses indicated no difference in support between one- and two-compartment models (Carleton et al. 2008; Martínez del Rio and Anderson-Sprecher 2008). Therefore, we used a first-order rate kinetic function of the form $y_t = y_\infty + (y_\infty - y_0)e^{-t/\tau}$ to model changes in FA carbon isotope values over time, where y_t is the sample $\delta^{13}C$ of a given FA at time t in parts per thousand, y_∞ is the estimated asymptotic $\delta^{13}C$ value of the FA when it has come into equilibrium with the second diet in parts per thousand, y_0 is the estimated $\delta^{13}C$ value of the FA at the time of the diet shift in parts per thousand, t is the measured time since the diet shift in days, and τ is the mean carbon retention time of the compartment in days, which reflects the rate of carbon turnover for a given FA. We used a model of the same form to test for changes in concentration of each FA over time, with τ representing the reciprocal of the instantaneous rate of change in concentration. In the one case where FA composition significantly changed over time (neutral fraction oleic acid, 18:1n9), we incorporated this into our estimate of carbon turnover by using the modified function $y_t = y_\infty + (y_\infty - y_0)e^{-t(k_g + k_d)}$ (Hesslein et al. 1993; Martínez del Rio and Carleton 2012), where k_g is the instantaneous rate of growth in the concentration of oleic acid, estimated above, and k_d is the rate of catabolic turnover. For this case, we report $1/k_d$, the equivalent of τ in the original function.

We used a two-step process to fit these functions to our data. First, we estimated average parameters for FA fractions (neutral and polar) with nonlinear least squares (R base package nls). Second, we used those estimates as starting values for nonlinear mixed effect models (nlme package) that included fixed effects to estimate parameters representing the differences in turnover between linoleic acid and other FAs and a random effect to account for individual variation among birds at the time of the diet shift. We then used multiple comparisons with a Bonferroni correction (polar df = 1, 354; neutral df = 1, 236) to test for differences between FAs not included as parameters in the mixed models. We repeated this process to test for effects of exercise on individual FAs: we first estimated average parameters for each FA in each fraction and then used those estimates as starting values for mixed effects models that included treatment group as a fixed effect and individual bird as a random effect. We used significance tests of turnover parameters to confirm the effect of exercise treatments on turnover. We did not include sex in our analyses, as we previously found no effect of sex on lipid turnover (Carter et al. 2018). We calculated discrimination factors ($\Delta^{13}C_{tissue\text{-}diet}$) for individual FAs by subtracting C_3 diet FA $\delta^{13}C$ values from the estimated asymptotic $\delta^{13}C$ values produced by the mixed models for individual FAs described above.

Results

FA Composition

Many of the same FAs were present in both the neutral and the polar fractions of lipids extracted from our flight muscle samples (fig. 2). Both fractions contained similar proportions of 16:0, while the polar fraction contained larger proportions of 18:0 and the long-chain PUFAs 20:4n6 and 22:6n3, and the neutral fraction had larger proportions of 18:1n9 and 18:2n6. FA composition was consistent over time for both lipid fractions and for all FAs, except for an increase in 18:1n9 concentration in the neutral fraction (fig. S1; figs. S1 and S2 are available online), described by the equation $y_t = 30.74 - (30.74 - 24.90)e^{-0.039t}$. By this estimate, the proportion of 18:1n9 in neutral lipid samples would reach 95% of its asymptotic value in 77.2 d.

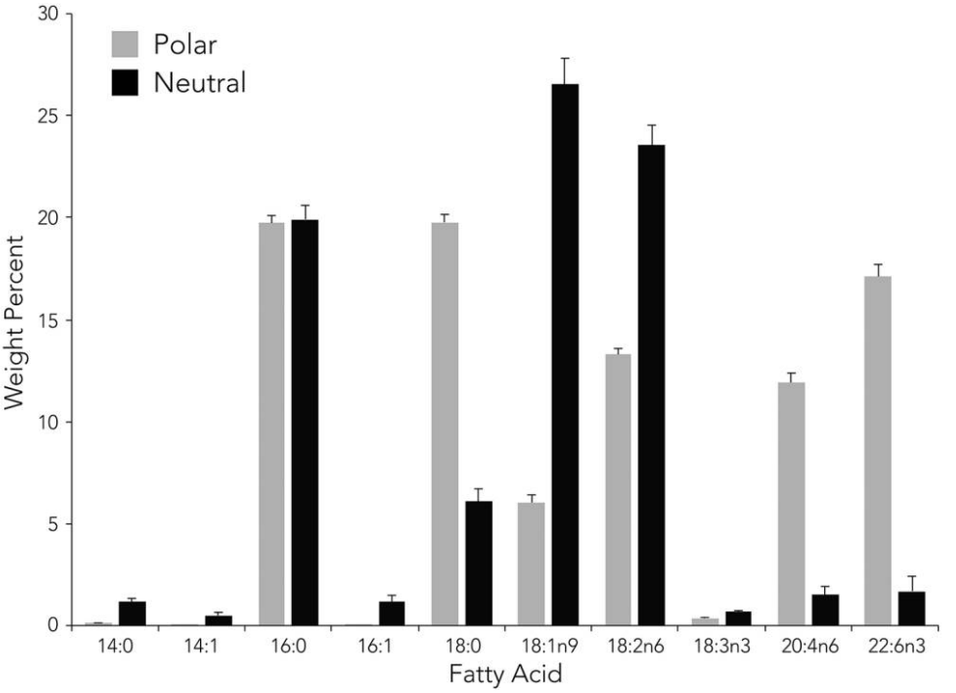

Figure 2. Concentrations of the 10 most common fatty acids in zebra finch pectoral muscle neutral and polar lipid fractions, averaged across time points. All fatty acid concentrations were consistent over time except 18:1n9 in the neutral fraction—the mean depicted here is intermediate between initial and equilibrium concentrations. Values are mass percent ± SE.

Carbon Isotopic Turnover of FAs

We measured δ^{13}C values and estimated turnover for four FAs in both the neutral and the polar fractions (16:0, 18:0, 18:1n9, and 18:2n6) and two additional FAs (20:4:n6 and 22:6n3) in the polar fraction (figs. 3, 4, S2). Turnover in the polar fraction was fastest for 18:2n6 ($\tau = 4.1 \pm 0.4$ d; mean ± SE), followed by 16:0 ($\tau = 5.1 \pm 0.4$), 18:1n9 ($\tau = 5.4 \pm 0.6$), 18:0 ($\tau = 11.7 \pm 1.2$), 20:4n6 ($\tau = 26.0 \pm 3.0$), and 22:6n3 ($\tau = 41.4 \pm 6.0$). Mean retention times (τ) significantly differed ($P < 0.05$) among these FAs except between the pairs of 18:2n6 and 16:0 ($P = 0.083$, $T_{354} = 1.740$) and 16:0 and 18:1n9 ($P = 0.669$, $T_{354} = -0.429$). Similarly, turnover in the neutral fraction was also fastest for 18:2n6 ($\tau = 6.6 \pm 1.0$, mean ± SE), followed by 16:0 ($\tau = 9.5 \pm 1.5$), 18:0 ($\tau = 9.5 \pm 1.5$), and 18:1n9 ($\tau = 23.6 \pm 7.7$). These turnover rates again significantly differed, except between the pairs of 18:2n6 and 16:0 ($T_{239} = 1.929, P = 0.055$) and 16:0 and 18:0 ($T_{239} = 0.035, P = 0.514$). The estimated mean retention time of 18:1n9 in the neutral fraction was increased by the inclusion of the term for fractional net growth, with an overall mean retention time of 12.3 ± 2.1 d.

The influence of exercise on turnover rates differed between fractions. In the polar fraction, turnover was significantly faster in the exercised group than in the sedentary control group for 18:2n6 ($\tau_{\text{exercised}} = 3.7 \pm 0.8, \tau_{\text{control}} = 5.5 \pm 0.7; P = 0.029, T_{56} = -2.245$; fig. 4*A*) and 16:0 ($\tau_{\text{exercised}} = 3.8 \pm 0.5, \tau_{\text{control}} = 5.3 \pm 0.6; P = 0.024, T_{59} = -2.314$), with similar trends for 18:1n9 ($\tau_{\text{exercised}} = 3.8 \pm 1.2, \tau_{\text{control}} = 5.8 \pm 1.1; P = 0.104, T_{58} = -1.663$) and 20:4n6 ($\tau_{\text{exercised}} = 21.1 \pm 3.1, \tau_{\text{control}} = 31.3 \pm 4.9; P = 0.102, T_{51} = -1.663$). In contrast, for the neutral fraction, there were no significant differences between exercised and control groups (fig. 4*B*). The variability in our estimates of mean retention time were considerably different across FAs and fractions, being greatest for 20:4n6 and 22:6n3 in the polar fraction and 18:1n9 in the neutral fraction.

Tissue-Diet Discrimination Factors

Birds were in isotopic equilibrium with their diet by day 256 after the diet switch, allowing us to quantify tissue-diet discrimination between estimated asymptotic δ^{13}C values and diet for four FAs: 16:0, 18:0, 18:1n9, and 18:2n6 (table 1). Discrimination differed among FAs, with 18:0, 18:1n9, and 18:2n6 exhibiting no discrimination for either fraction. In contrast, 16:0 had a positive discrimination in the polar fraction ($\Delta^{13}C = 1.4 \pm 0.3$; 95% confidence interval [CI]: 0.81–1.99), while the δ^{13}C of neutral fraction 16:0 was not significantly different from either the diet or polar fraction 16:0 but did trend away from diet 16:0 ($\Delta^{13}C = 1.0 \pm 0.5$; 95% CI: −0.07–2.07).

Discussion

FA Composition

The FA profiles of muscle lipids in our zebra finches were largely consistent with previous studies on other songbirds

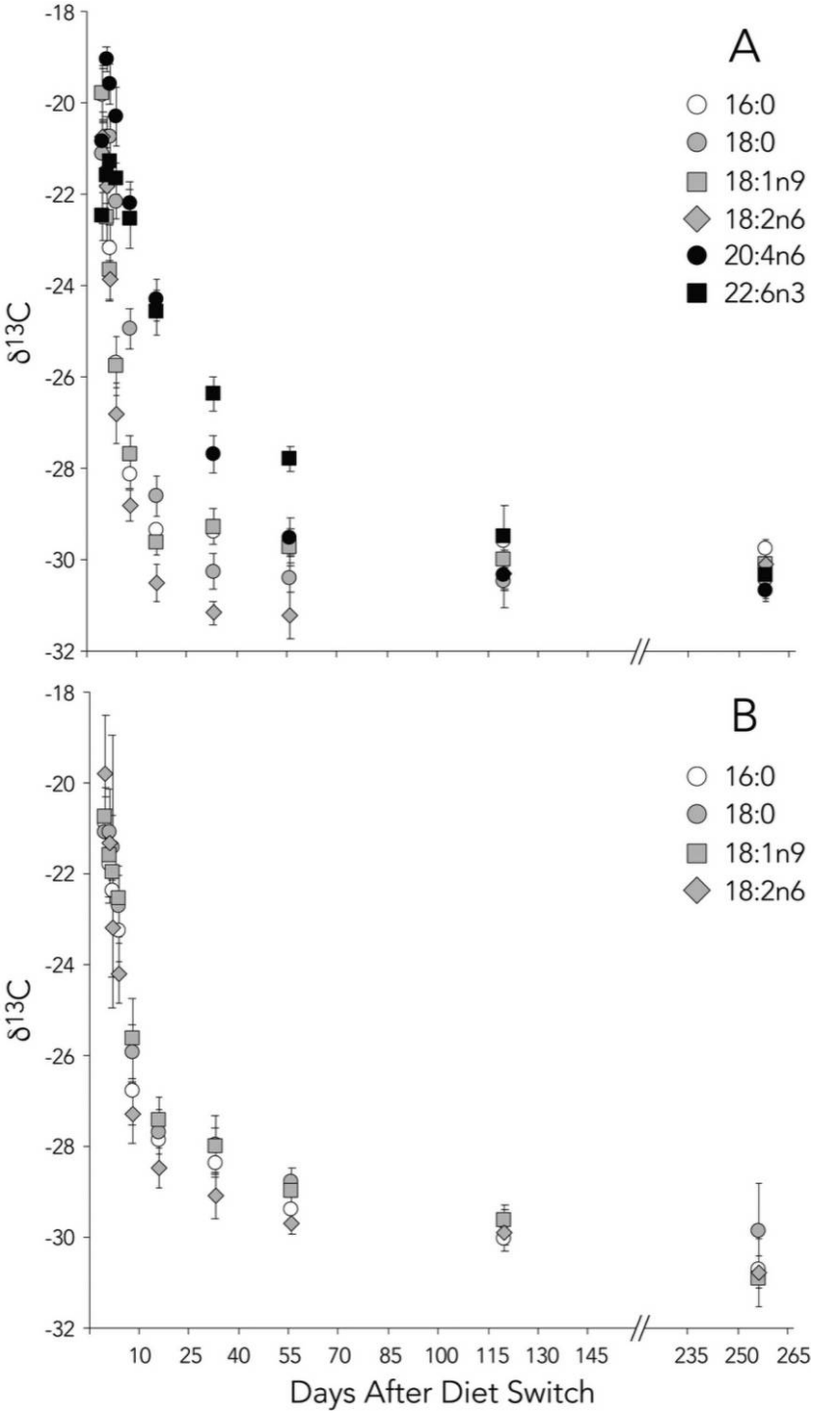

Figure 3. Mean $\delta^{13}C$ values (±SE) of fatty acids in the polar (*A*) and neutral (*B*) fractions of zebra finch flight muscle lipids, sampled at 10 time points following a shift from a C_4- to a C_3-based diet. The *X*-axis has been truncated to reflect the time gap in samples between days 120 and 256 and to highlight the rate of change in $\delta^{13}C$ values soon after the diet shift.

(Pierce et al. 2005; Klaiman et al. 2009; McCue et al. 2009), with predominant FA in the neutral fraction including 16:0, 18:1n9, and 18:2n6 and in the polar fraction also including 18:0, 20:4n6, and 22:6n3 (fig. 2). Saturated (16:0) and monounsaturated (18:1n9) FAs are synthesized from dietary carbohydrates and proteins to allow efficient storage of dietary energy (Klasing 1998), which explains their dominance in the neutral fraction. Conversely, PUFAs are more associated with

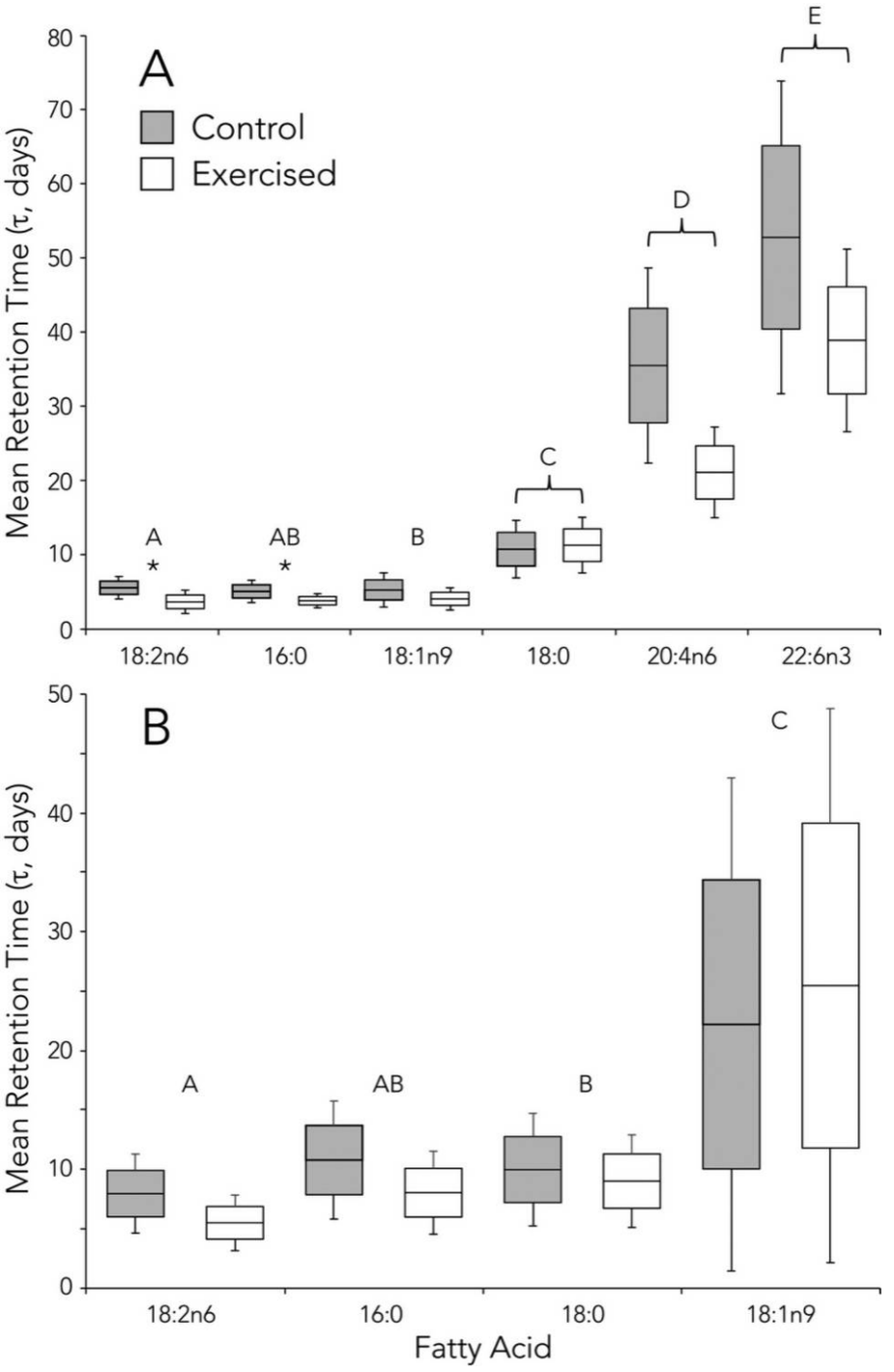

Figure 4. Estimated mean retention times (τ, days) of fatty acids (FAs) in the polar (*A*) and neutral (*B*) fractions of flight muscle lipids collected from exercised and control zebra finches. Boxes depict 25% and 75% confidence intervals, with whiskers representing 95% confidence intervals. Mean retention time significantly differed between FAs with different letters, and asterisks indicate significant differences between exercised and control groups.

cell and organelle membranes (Stubbs and Smith 1984; Infante et al. 2001; Turner et al. 2006), leading to the high concentrations of 18:2n6, 20:4n6, and 22:6n3 in the polar fraction. FA composition in both fractions was consistent across exercise groups and largely stable over time after the diet shift, with only 18:1n9 concentration in the neutral fraction significantly increasing over time (fig. S1). This change may have been driven by a probable increase in dietary 18:1n9 (C_4 diet

Table 1: Equilibrium δ^{13} values and estimated tissue-diet discrimination factors (Δ^{13}C) for neutral and polar fractions of pectoral muscle lipids

	16:0	18:0	18:1n9	18:2n6
δ^{13}C:				
C_3 diet	−31.1 ± .2^{A}	−30.4 ± .4	−29.5 ± .1	−30.1 ± .3
Neutral	−30.1 ± .5^{AB}	−29.4 ± .5	−29.6 ± .6	−30.3 ± .5
Polar	−29.7 ± .2^{B}	−30.7 ± .4	−29.8 ± .4	−30.7 ± .4
Δ^{13}C:				
Neutral	1.0 ± .5	1.0 ± .7	−.1 ± .6	−.2 ± .6
Polar	**1.4 ± .3**	−.3 ± .6	−.3 ± .4	−.6 ± .5

Note. Values are presented as mean ± SE. All values are in parts per thousand. The boldface discrimination value highlights a significant positive discrimination factor for 16:0 in the polar fraction. Where present, different superscript letters indicate significant differences in equilibrium δ^{13}C enrichment.

[24.3%] vs. C_3 diet [29.8%]; table S1). However, a shift of similar magnitude in dietary availability of 16:0 (C_4 [17.0%] vs. C_3 [11.4%]) did not cause a similar change in bird tissue. Alternately, the slightly elevated protein content of our C_3 diet (15%) relative to C_4 diet (11%) could have increased the available pool of substrate for de novo synthesis and storage of 18:1n9 (Bairlein 1998; Stevens 2004), although increased de novo synthesis of 18:1n9 without corresponding increases in 16:0 and 18:0 is highly unlikely.

FA Turnover

In both polar and neutral fractions, 18:2n6 and 16:0 turned over the most quickly; FAs with the slowest turnover differed between fractions: 18:1n9 for neutral and the long-chain PUFAs 20:4n6 and 22:6n3 for polar. There are several factors that could influence the turnover of FAs and result in these patterns (fig. 1; table 2). First, all else being equal, measured turnover will be dependent on tissue concentration: abundant FAs will have slower turnover than rare FAs (Hesslein et al. 1993; Martínez del Rio and Carleton 2012; Salini et al. 2016). However, the synthesis of 18:0, 18:1n9, and 20:4n6 from other FAs (Stevens 2004; Schmitz and Ecker 2008) and the incorporation of carbon from nonlipid sources during de novo synthesis (Yoo et al. 2004) could increase the tissue concentration of some FAs. Accordingly, our baseline expectation was for turnover among neutral lipids to be fastest in 18:2n6, followed by 16:0, 18:0, and 18:1n9, and for turnover among polar lipids to be fastest in 18:2n6, followed by 22:6n3, 20:4n6, 16:0, 18:0, and 18:1n9 (table 2). Second, the higher dietary concentrations of 18:2n6, 18:1n9, and 16:0 (table S1) could result in more rapid dilution of those FA pools in muscle

Table 2: Predicted and observed rank ordering of fatty acid turnover rates and presence of exercise effects

		Predicted ranking based on different influences			
Fatty acid fraction and turnover rate	Observed	Adjusted tissue concentration	Diet concentration	Oxidative damage	Functional catabolism
Neutral:					
Fastest	18:2n6	18:2n6	18:2n6	18:2n6	18:2n6
↓	16:0	16:0	18:1n9	16:0	16:0
	18:0	18:0	16:0	18:0	18:1n9
Slowest	18:1n9	18:1n9	18:0	18:1n9	18:0
Polar:					
Fastest	18:2n6	18:2n6	18:2n6	22:6n3	20:4n6
↓	16:0	22:6n3	18:1n9	20:4n6	22:6n3
	18:1n9	20:4n6	16:0	18:2n6	18:2n6
	18:0	16:0	18:0	16:0	16:0
	20:4n6	18:0	20:4n6	18:0	18:0
Slowest	22:6n3	18:1n9	22:6n3	18:1n9	18:1n9
Exercise effects?	Neutral: no Polar: yes	No	No	Yes	Yes

Note. Predictions are based on the hypothesized effects of diet fatty acid concentration, tissue fatty acid concentration adjusted for interconversion between certain fatty acids, oxidative damage, and functional catabolism (see main text for rationale). Ranks are from fastest to slowest.

tissue (table 2), while the absence of dietary 20:4n6 and 22:6n3 could result in slower turnover. However, given the stability of muscle FA composition, the routing of dietary FAs to tissue pools must have been balanced by catabolism of those same FAs. Third, turnover rate should increase with the rate at which FAs suffer oxidative damage and are removed from membranes or lipid droplets. Susceptibility to damage in FAs is closely related to degree of unsaturation (Mataix et al. 1998; Hulbert 2010; Skrip and McWilliams 2016), leading to faster turnover of polar fraction long-chain PUFAs (table 2). Finally, turnover rate could be driven by the catabolism of FAs for specific functions. The mobilization rates of FAs for oxidation during energy metabolism are often associated with decreasing chain length and increasing desaturation (Raclot 2003; Price et al. 2008), so we would expect relatively faster turnover of neutral fraction 18:1n9 (table 2). For polar lipids, a likely use is the synthesis of signaling molecules derived from long-chain PUFAs stored in lipid membranes (Zhou and Nilsson 2001; Marion-Letellier et al. 2016), potentially resulting in faster turnover of 20:4n6 (table 2). In addition, oxidative damage and functional catabolism likely increase with elevated metabolic rate, which has been associated with greater production of damaging reactive species (Mataix et al. 1998; Jenni-Eiermann et al. 2014), increased catabolism of neutral fraction energy stores (Jenni and Jenni-Eiermann 1998; McClelland 2004), and higher circulating levels of some lipid-derived hormones (Chen et al. 1993). Thus, we expect faster FA turnover in exercised birds than in sedentary birds if either of these mechanisms is driving FA turnover (table 2).

Of these factors, turnover in the neutral fraction was most consistent with predictions based on tissue concentration (table 2). The slow and variable turnover of 18:1n9 is inconsistent with predictions based on consumption for energy production and diet concentration, and the lack of response to exercise suggests that (1) increased fuel demands are met by nonmuscular sources via birds' multifaceted lipid-transport system (Jenni and Jenni-Eiermann 1998; Guglielmo 2010) and (2) intramuscular triglycerides are not exposed to substantial risk of oxidative damage. These results also emphasize the interconnection between FA pools, enabling a small pool such as 18:0 to exhibit relatively slow turnover.

In comparison to the neutral fraction, turnover in the polar fraction was more complex (table 2). The slower turnover observed in 20:4n6 and 22:6n3 was consistent only with the predictions of diet concentration and suggests that songbirds (1) prevent substantial oxidative damage to long-chain PUFAs and (2) preferentially retain them in membranes to balance their limited availability in seed-based diets. Domains of membranes could be protected from oxidative damage by the inclusion of antioxidants such as cholesterol or vitamin E in membranes (Mataix et al. 1998; Samuni et al. 2000). The apparent retention of 20:4n6 and 22:6n3 may be the result of a shift from de novo phospholipid synthesis to deacylation-reacylation reactions (Chakravarthy et al. 1986; Kuwae et al. 1997), which rely on molecule-specific enzymes (Contreras et al. 2001) that could result in selective recycling of poorly provisioned FAs. In addition, metabolically complex PUFAs (e.g., 22:6n3) may be less likely to be catabolized because such breakdown requires multiple steps, including several in peroxisomes (Madsen et al. 1999).

Surprisingly, the fast turnover of polar fraction 18:2n6, 16:0, and 18:1n9 was similar to the predicted turnover for the neutral fraction based on energy consumption (table 2). This suggests that some phospholipid-derived FAs are used as an energy source. Such use would also be consistent with the significant increase in turnover of 18:2n6 and 16:0 and the trending increase in 18:1n9 that we observed in polar fraction lipids of exercised birds. Both this consumption and the recycling of long-chain PUFAs could occur simultaneously as damaged mitochondria are degraded in peroxisomes. Finally, faster turnover of 20:4n6 in exercised birds could indicate elevated release of n-6 PUFA-derived eicosanoid hormones from muscle in response to increased metabolic rate, perhaps mediating an inflammation response to exercise (Watkins 1991; Ronni-Sivula et al. 1993; Boger et al. 1995; Schmitz and Ecker 2008; Price 2010).

Turnover was faster in the polar fraction than in the neutral fraction for 18:2n6, 16:0, and most notably 18:1n9 (figs. 3, 4, S2), which is consistent with our finding of faster turnover of bulk polar lipids than bulk neutral lipids (Carter et al. 2018). The smaller tissue concentrations of 18:1n9 and 18:2n6 (fig. 2) and elevated catabolism (as evidenced by exercise effects; fig. 4) likely explain the faster turnover of the polar fraction.

FA Tissue-Diet $\delta^{13}C$ Discrimination

For lipids, the most substantial isotopic fractionation is the depletion of ^{13}C during de novo synthesis from acetyl CoA produced from glycolysis (DeNiro and Epstein 1977), although fractionation during catabolism may explain ^{13}C enrichment for some lipids relative to those in the diet (Budge et al. 2011; Ben-David et al. 2012). However, only one FA had significant $\delta^{13}C$ discrimination relative to those in the diet (table 1): 16:0 exhibited a 1.4‰ and 1.0‰ increase in the polar and neutral fractions, respectively, although the latter was not significantly different from zero. While the observed positive tissue-diet $\delta^{13}C$ discrimination of polar fraction 16:0 would be consistent with the selective catabolism of lighter molecules, such catabolism would be inconsistent with the rapid turnover of this FA, which corresponds to a rapid accumulation of lighter molecules. Alternately, it is possible that tissue 16:0 is largely synthesized de novo, decoupling it from dietary 16:0 and resulting in isotopic discrimination. In either case, it is unclear why this effect would be stronger in the polar fraction.

The lack of $\delta^{13}C$ discrimination of 18:0, 18:1n9, and 18:2n6 in either fraction may indicate that these FAs are predominantly routed directly from diet, although in each case there was relatively high variability around the estimates of the $\delta^{13}C$ value of the asymptote. For 18:0 and 18:1n9, this high variability at equilibrium may suggest that individuals differ in the degree of de novo lipid synthesis that they undertake, whereas for 18:2n6 it likely suggests selective oxidation or further metabolic processing in some individuals. Overall, these results in-

dicate that there is no single pattern of isotopic discrimination of FAs, but instead multiple mechanisms are important, each dependent on FA supply versus demand and functional use.

Implications for Songbird Ecology

Our results have several important implications for songbird ecology. First, overall lipid turnover was fast. Specifically, mean retention times for 18:2n6, 16:0, and 18:1n9 in the polar fraction were less than 1 wk; 50% of these FAs turn over in 3 or 4 d, and 95% turn over in <18 d. Thus, by changing diets, songbirds could substantially remodel their cellular membranes during a single migration stopover (Schaub and Jenni 2001; Seewagen and Guglielmo 2010; Cohen et al. 2014). Turnover in the neutral fraction was slower than in the polar fraction, but on average, both 18:2n6 and 16:0 achieved 95% replacement in <28 d, which could allow substantial changes in FA composition during preparation for migration, reproduction, or other energy-intensive activities.

Second, both functionally important long-chain PUFAs in this study (20:4n6 and 22:6n3) had slow turnover, with 95% replacement requiring >70 d. The long residence times and corresponding low demand for these PUFAs suggest that their low dietary concentrations are not a limitation for adult songbirds using terrestrial resources, which can likely synthesize long-chain PUFAs from their precursors at relatively high rates (Watkins 1991; Käkelä et al. 2009; Twining et al. 2016*a*). This contrasts with evidence of limitation in insectivorous songbirds that rely on PUFA-rich aquatic resources and lack the ability to elongate and desaturate shorter essential FAs into long-chain PUFAs (Martínez del Rio and McWilliams 2016; Twining et al. 2016*b*). Similarly, long-chain PUFA limitation may be more important to nestlings, for whom protection and recycling would be insufficient to meet their needs during rapid tissue accretion.

Finally, our estimates of turnover lead to novel estimations of the FA supply needed by a songbird to maintain a consistent FA composition. Based on lipid content and FA concentrations of our samples, zebra finches in our study averaged 3.6 mg of neutral and 2.0 mg of polar fraction 18:2n6 per gram of wet muscle tissue. With fractional incorporation rates ($1/\tau$) of 0.11 and 0.24, respectively, these birds needed to assimilate 0.88 mg of 18:2n6 per gram of muscle per day to maintain their FA composition; this requires a dietary FA concentration of at least 1.3 mg/g (assuming total food intake of 1.5 g per day; Bauchinger et al. 2010). This requirement is for the pectoralis muscle only and is likely higher than in larger-sized species due to the allometric scaling of turnover (Bauchinger and McWilliams 2009; Salini et al. 2016). To place this in an ecological context, grains and fruits commonly eaten during migration contain sufficient concentrations of 18:2n6 (e.g., 5.8 mg/g in *Viburnum dentata*, 15.9 mg/g in *Parthenocissus quinquefolia*; Zygadlo et al. 1995; Smith et al. 2007; McCue et al. 2009; Patterson and Magnuson 2014; Pierce and McWilliams 2014). Given the increase in turnover in our exercised birds, this requirement is likely to increase during migration, but concurrent hyperphagia or selective routing of 18:2n6 from adipose stores could allow birds to meet elevated demands. Such estimates are an important step in linking songbird physiology and ecology and emphasize the informative value of turnover rate studies.

Acknowledgments

We thank Barbara Pierce for providing us with the zebra finches used in this experiment and for the use of her GC-FID for fatty acid analyses. We also thank Luke Douglas, Megan Grey, and Lara Kazo for their assistance with animal care and Megan Skrip for the refinement of our animal care and flight training protocols. Finally, we thank Laura Burkemper and Emma Elliott-Smith for their assistance with the gas chromatography–combustion–isotope ratio mass spectrometry. Funding for this study was provided by the National Science Foundation (IOS-0748349) and the US Department of Agriculture (RIAES-538748).

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