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RESEARCH ARTICLE

Turnover of muscle lipids and response to exercise differ between neutral and polar fractions in a model songbird, the zebra finch

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ABSTRACT

The turnover rates of tissues and their constituent molecules give us insights into animals' physiological demands and their functional flexibility over time. Thus far, most studies of this kind have focused on protein turnover, and few have considered lipid turnover despite an increasing appreciation of the functional diversity of this class of molecules. We measured the turnover rates of neutral and polar lipids from the pectoralis muscles of a model songbird, the zebra finch (*Taeniopygia guttata*, *N*=65), in a 256 day C_3/C_4 diet shift experiment, with tissue samples taken at 10 time points. We also manipulated the physiological state of a subset of these birds with a 10 week flight training regimen to test the effect of exercise on lipid turnover. We measured lipid $\delta^{13}C$ values via isotope ratio mass spectrometry (IRMS) and estimated turnover in different fractions and treatment groups with non-linear mixed-effect regression. We found a significant difference between the mean retention times (τ) of neutral and polar lipids (t_{119}=−2.22, *P*=0.028), with polar lipids (τ=11.80±1.28 days) having shorter retention times than neutral lipids (τ=19.47±3.22 days). When all birds were considered, we also found a significant decrease in the mean retention time of polar lipids in exercised birds relative to control birds (difference=−2.2±1.83 days, t_{56}=−2.37, *P*=0.021), but not neutral lipids (difference=4.2±7.41 days, t_{56}=0.57, *P*=0.57). A larger, more variable neutral lipid pool and the exposure of polar lipids in mitochondrial membranes to oxidative damage and increased turnover provide mechanisms consistent with our results.

KEY WORDS: Lipid turnover, Carbon pools, Energy metabolism, Flight training

INTRODUCTION

Turnover rates of key constituents that comprise an organism have been widely used to understand the dynamics of resource pools relevant to biological systems, such as body water (Lifson and McClintock, 1966; Speakman, 1997), hormones and neurotransmitters (Brodie et al., 1966; Weinstock, 2001), and tissue carbon (Carleton and Martínez del Rio, 2005; Hobson and Clark, 1992a; Tieszen et al., 1983), and to gain insights into how diet and activity affect processes that occur at the tissue and cellular levels (Bauchinger and McWilliams, 2009; Bauchinger et al., 2010; Boom et al., 1996; Cruz et al., 2005). Furthermore, knowledge of the dynamics of these key constituents can also be used to integrate molecular processes at the cellular and tissue level over time into outcomes relevant at the organism level. For example, turnover rate determines the pace at which organs within organisms can change, thus determining how quickly their form and function can change and defining the extent of phenotypic flexibility of the system (Bauchinger and McWilliams, 2010; Piersma and Lindström, 1997). Thus, estimating the turnover rates of resource pools is an important step towards mechanistically linking processes at the organism, tissue and cellular levels.

Lipids are important constituents of animal tissues, comprising more than half the dry mass of some tissues and serving vital functions as the primary component of cell membranes, energy storage molecules and signaling molecules (Klasing, 1998; Stevens, 2004). Furthermore, there is a growing appreciation of the importance of fatty acid composition to the functionality of lipid-based tissues and tissue components (Pierce and McWilliams, 2014; Price, 2010). Much of this research has focused on birds because of their greater reliance, relative to mammals and other vertebrates, on lipids for energy metabolism (Blem, 1976; Butler and Woakes, 1990; Gudmundsson et al., 1990; Guglielmo, 2010; McWilliams et al., 2004). Despite the potential importance of lipid pool dynamics for understanding the relationship between fatty acid composition and organism-level outcomes, there have been few attempts to quantify the turnover of lipids, and even fewer in birds, with most studies instead focusing on protein turnover (Bauchinger and McWilliams, 2009; Carleton and Martínez del Rio, 2005; Hobson and Yohannes, 2007; MacAvoy et al., 2006; Tieszen et al., 1983). For example, studies of lipid-free carbon turnover have found up to threefold difference in the turnover rates of different tissues, hypothesized to result from either the metabolic rate of the tissue (Klaassen et al., 2004; MacAvoy et al., 2006) or fundamental differences in protein synthesis (Bauchinger and McWilliams, 2009; Carleton et al., 2008). Therefore, the primary objective of this study was to estimate the turnover of lipids in a model songbird, the zebra finch (*Taeniopygia guttata*) and the effect of metabolic rate on lipid turnover, by quantifying the turnover of the ^{13}C stable isotope following the shift from a C_3 to a C_4 diet (Tieszen et al., 1983) in exercised and sedentary birds.

The pool of all lipids in an animal can be largely separated into two major fractions: neutral lipids (NLs, e.g. triacylglycerols) and polar lipids (PLs, e.g. phospholipids) (Stevens, 2004). Given the broad separation of functional roles for these fractions, with NLs primarily used in energy metabolism and PLs in membranes, we might expect turnover to differ between them. As NLs are consumed for fuel, they must be replaced to maintain a consistent pool, so turnover rate should be directly related to the rate of fuel use and indirectly related to metabolic rate (Jenni and Jenni-Eiermann, 1998). In contrast, PLs are unlikely to be metabolized at high rates, but their location in membranes, particularly those of the mitochondria, would expose them to greater amounts of damage from the reactive species (RS) that are byproducts of energy metabolism (Gaál et al., 2006; Hulbert, 2010; Mataix et al., 1998;

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Samuni et al., 2000; Wong-ekkabut et al., 2007). Damage would then necessitate replacement, thus directly linking PL turnover to metabolic rate. A study in rats found 10–30% slower turnover in the PLs of the heart than in plasma lipids (Wahjudi et al., 2011), but this has the limitation of comparing across tissues. We compared turnover rates of NL and PL fractions in pectoral muscle of exercised and unexercised adult male and female zebra finches to avoid the confounding effects of comparisons between tissues. An additional objective of this study was to estimate the ^{13}C discrimination factors between diet components and tissue lipids when they had reached equilibrium. These discrimination factors are essential for using stable isotope data to reconstruct dietary information for wild animals (Barquete et al., 2013; Hobson and Bairlein, 2003; Hobson and Clark, 1992b)

MATERIALS AND METHODS

Animal care and maintenance

All care and experimental procedures were reviewed and approved by the University of Rhode Island's Institutional Animal Care and Use Committee under protocol AN11-12-009. We randomly sorted 65 adult zebra finches, *Taeniopygia guttata* Vieillot 1817, into two experimental groups, control (*N*=32, 15 males, 17 females) and exercised (*N*=33, 16 males, 17 females), and housed them in four single-sex and experimental group aviaries (2.2 m×1 m×2 m). We maintained the room on a 12 h:12 h light:dark schedule and at an air temperature of 23–27°C throughout the experiment. Birds had access to *ad libitum* food, water, mineral-enriched grit and cuttlebone except during the period of daily flight training described below. For the first 90 days of the experiment we fed birds a C_4 mixed-seed diet (−15.57±0.34‰ bulk $\delta^{13}C$, Hagen no. B2405, Mansfield, MA, USA) primarily composed of millet (*Pennisetum glaucum*) to enrich tissues with ^{13}C. Following this enrichment period, we switched birds to a C_3 mixed-seed diet (−27.70±0.01‰ bulk $\delta^{13}C$, Abbaseed no. 3700, Hillside, NJ, USA) primarily composed of canary grass (*Phalaris canariensis*) for the remainder of the experiment. We measured fat score on a 0–7 scale (no visible fat to complete coverage, respectively; adapted from Eck et al., 2011) and body mass to the nearest 0.1 g weekly for the duration of the experiment to monitor bird health.

Experimental treatments and tissue samples

We began flight training 2 weeks prior to the diet shift (day −14) and continued the training daily for 10 weeks, until day 56 relative to the diet shift. For the exercised group, flight training consisted of 2 h of total flying time each day, divided into two 1 h sessions starting at 11:00 h and 13:30 h, for the full 10 week period. During flight training, birds flew as a flock around a 6 m×3 m×2 m flight arena with perches in opposite corners and a 4 m partition running lengthwise through the center of the arena. A handler continuously walked clockwise around the arena during flight training sessions for 300 laps every hour resulting in 1200 flights of at least 7 m from perch to perch for each bird each day, or approximately 8.5 km per day. The energetic costs of this type of short-burst flight are approximately three times higher than those of sustained flight for small songbirds (Nudds and Bryant, 2000). Additionally, this specific flight training regimen has also demonstrably increased metabolic rates as evidenced by an average increase of 80% in apparent metabolizable energy intake (Bauchinger et al., 2010). Concurrent with flight training, we removed food and water from birds in the control (sedentary) group so that exercised and control birds were without food for the same period.

On days 0, 1, 2, 4, 8, 16, 33, 56, 120 and 256 (relative to the diet shift), we took 2–4 birds from each experimental group (1–2 birds of each sex), decapitated them (see below) and immediately extracted the pectoral muscle. Other internal organs were sampled and stored for later analysis. Sampling on day 0 immediately preceded the diet shift, so those birds had only been fed the C_4 diet. More frequent sampling of birds soon after the diet shift is necessary to capture the period of greatest change in carbon isotopic values, whereas samples on days 120 and 256 provided estimates of the asymptotic carbon isotopic values (Bauchinger and McWilliams, 2009, 2010). On a given day, all sampled birds were placed in cloth bags immediately after flight training, weighed to the nearest 0.1 g and assessed for fat score. They were decapitated within 80 min of capture and the pectoral muscles and other selected organs were subsequently removed within 10 min. Tissue samples were 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.

Lipid extraction and stable isotope analysis

We extracted total lipids from pectoral samples using a modified Folch method (Folch et al., 1957; Guglielmo et al., 2002a). Briefly, approximately 400 mg of wet tissue was homogenized in 6 ml of 2:1 chloroform:methanol with a high-speed stainless steel homogenizer (PowerGen 700, Fisher Scientific, Waltham, MA, USA), centrifuged at 3000 rpm for 15 min, and then aqueous solutes were separated by rinsing with 2 ml of 0.25% KCl. The organic phase was carefully removed with a Pasteur pipette and transferred to a 4 ml glass vial where it was dried under N_2 and resuspended in 200 µl chloroform. The fatty acid/chloroform solution was then transferred to Supelclean solid phase extraction columns (Supelco, LC-NH_2, 1 ml aminopropyl bonding) conditioned with 2 ml hexane, and NL, non-esterified fatty acid (NEFA) and PL fractions were sequentially eluted with 1.8 ml 2:1 chloroform:isopropanol, 2 ml 49:1 isopropyl ether: acetic acid and 2 ml methanol. NL and PL fractions were collected in 4 ml vials while the NEFA fraction was discarded. To focus on the turnover of fatty acids and facilitate comparisons with parallel analyses of compound-specific turnover, fractionated samples were then esterified into fatty acid methyl esters (FAMEs) by drying under N_2, resuspending in 1 mol l^{-1} acetyl chloride in methanol, and heating at 90°C for 2 h. We ground diet samples with a Wiley mill (no. 20 sieve; Thomas Scientific, Swedesboro, NJ, USA) and extracted and derivatized lipids from diet via the above protocol. Duplicate aliquots of FAMEs from both tissue and diet samples were measured into tin capsules, allowed to dry under N_2, and the ^{13}C enrichment of each fractionated sample was measured by continuous flow isotope ratio mass spectrometry (IRMS) using an Elementar Vario Micro Cube elemental analyzer interfaced to an Elementar Isoprime 100 Mass Spectrometer (Elementar Americas, Mt Laurel, NJ, USA). We also measured ^{13}C enrichment of aliquots of ground bulk diet and the non-lipid remainder of diet lipid extractions to compare $\delta^{13}C$ discrimination between tissue lipids and different diet components. Carbon isotope ratios were expressed as a per mil deviation ($\delta^{13}C$ ‰) from the reference standard Pee Dee Belemnite (PDB): $\delta^{13}C$ ‰=$[(R_{sample}/R_{PDB})-1]\times1000$, where $R=^{13}C/^{12}C$. Replicate FAME $\delta^{13}C$ measurements were averaged within individual birds and fractions and were then corrected to account for the addition of a methyl group by calculating the offset between a methylated and unmethylated palmitic acid standard.

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Table 1. AICc values, differences and weights for one- and two-compartment models of $\delta^{13}C$ isotopic incorporation of pectoral muscle neutral and polar lipid fractions of zebra finch (*Taeniopygia guttata*)

Fraction and model	No. of parameters	AICc	ΔAICc	Weight	Cumulative weight	log Likelihood
Neutral lipids						
One compartment	8	238.37	0.0	1	1	−109.90
Two compartment	12	249.01	10.7	0	1	−109.53
Polar lipids						
One compartment	8	182.47	0.0	0.79	0.79	−81.95
Two compartment	12	185.07	2.6	0.21	1.00	−77.53

AICc, Aikaike's information criterion, corrected for small sample size.

Carbon turnover and statistical analyses

As expected, carbon isotopic values displayed a pattern of exponential decay over time, thus requiring non-linear modeling to estimate carbon turnover. The standard exponential decay function is a one-compartment model with first-order rate kinetics of the form: $y_t=y_\infty+ae^{(-t/\tau)}$, where y_t is the measured $\delta^{13}C$ value of the sample at time t (‰), y_∞ is the estimated asymptotic $\delta^{13}C$ value of the tissue when it has come into equilibrium with the second diet (‰), a is the estimated difference between the $\delta^{13}C$ values of tissues at the time of the diet shift and at equilibrium with the second diet (‰), t is the measured time since the diet shift in days, and τ is the estimated mean carbon retention time of the compartment in days (a measure used to describe the rate of carbon turnover for a given tissue). However, it has also been suggested that multi-compartment models may more accurately describe the isotopic incorporation of some tissues (Carleton et al., 2008; Cerling et al., 2007; Martínez Del Rio and Anderson-Sprecher, 2008). Therefore, we fitted to our NL and PL carbon isotopic data both the one-compartment model described above and a two-compartment model of the form $y_t=y_\infty+a(p)e^{(-t/\tau_1)}+a(1-p)e^{(-t/\tau_2)}$, where p is the proportion of lipids in the first compartment and τ_1 and τ_2 are, respectively, the estimated mean carbon retention times of the first and second compartments.

We used a two-step process to fit both one- and two-compartment non-linear models, and then used the best-fit model to determine the effect of experimental group and sex on mean retention time. The first step involved estimating average parameters for NL and PL fractions with non-linear least-squares regression to use as starting values for step two. In the second step, we then used those estimated starting values for non-linear mixed-effect models that included fixed effects of experimental group and sex on mean retention time and a random effect to account for variable $\delta^{13}C$ starting points among birds at the time of the diet shift. Two-compartment models failed to converge when fitting all five parameters, but successfully converged when we replaced the y_∞ parameter with average $\delta^{13}C$ values from day 256. We therefore present the results from these four-parameter two-compartment models. To determine the best-fit models for NL and PL fractions, we compared AICc (Akaike's information criterion corrected for small sample size) values for one- and two-compartment models for each lipid fraction and selected the non-linear mixed-effect model with greater support (ΔAICc>2; Burnham and Anderson, 2002).

To test for differences in carbon turnover between NL and PL fractions, we pooled data from both fractions (N=130, an NL and PL sample from each bird) and then fitted the best-supported model (one- or two-compartment) via the procedure described above. In contrast with the models described above, lipid fraction was the only fixed effect included in this mixed-effects model.

To investigate factors that might explain differences in mean carbon retention time between fractions, we calculated residual $\delta^{13}C$ values for each individual and fraction from their appropriate best-fit model. Residuals were evenly distributed around zero throughout the experiment and met all statistical assumptions. We then tested for correlations between residuals and characteristics of individual birds including body mass, pectoral muscle mass, adipose tissue mass, liver mass, small intestine mass, fat score, lipid content of the pectoral muscles and percent of pectoral lipids in the neutral fraction. All statistical analyses were conducted in R (version 3.2.5, R Core Team, Vienna, Austria).

RESULTS

Carbon isotopic turnover

Carbon isotopic incorporation for both NL and PL fractions was best described by one-compartment models (Table 1), therefore these models were used to estimate mean retention time and test for differences between fractions and groups. Mean carbon retention time of pectoral muscle NL fractions was on average greater than that for the PL fractions (Fig. 1; t_{119}=−2.22, P=0.0279), with the NL fraction having a mean retention time of τ=19.5±3.2 days (mean± s.e.) and the PL fraction having a mean retention time of τ=11.8± 1.3 days. Mean carbon retention time in the PL fraction was significantly smaller in exercised birds (τ=10.5±1.4 days) than in control birds (τ=12.7±1.7 days; Figs 1 and 2; t_{56}=2.37, P=0.0212). However, this result was sensitive to the presence of a single statistical outlier (residual greater than 1.5 interquartile range) in the exercised group, removal of which increased the estimate of mean retention time (τ=11.2±1.2 days) and made the effect

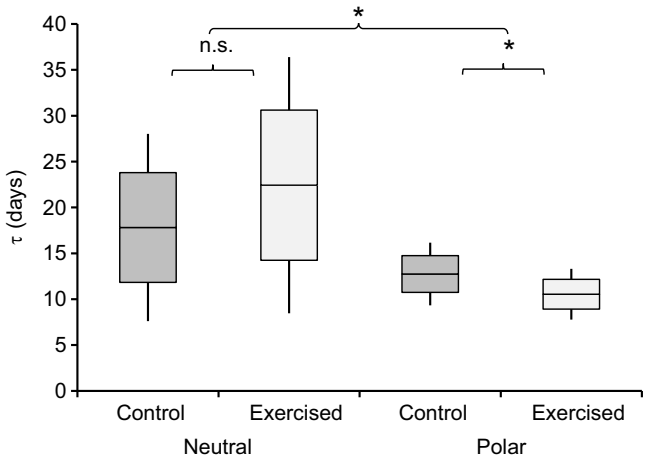

Fig. 1. Mean carbon retention time (τ) for neutral and polar fractions of the zebra finch (*Taeniopygia guttata*, *N*=65) pectoral muscle lipids. Boxplots depict mean, 25% and 75% confidence intervals, while error bars depict 5% and 95% confidence intervals. Asterisks indicate significant differences between fractions or treatment groups. Turnover was significantly faster in the polar fraction than in the neutral fraction (t_{119}=−2.22, P=0.0279, two-tailed), and turnover within the polar fraction was significantly faster in the exercised group (t_{56}=2.37, P=0.0212, two-tailed).

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Table 2. Estimated tissue–diet discrimination factors ($\Delta^{13}C$) for bulk diet, lipid-extracted diet and dietary lipids

	$\delta^{13}C$	$\Delta^{13}C$ diet – NL	$\Delta^{13}C$ diet – PL
Bulk diet	–27.70±0.01	3.41±0.172	3.74±0.169
Lipid-extracted diet	–26.89±0.01	4.22±0.172	4.55±0.169
Dietary lipids	–31.23±0.01	–0.12±0.172	0.21±0.169

Estimates are presented as means±s.e. NL, neutral lipid; PL, polar lipid.

Table 3. Correlation coefficients between measures of fat storage and tissue composition of individual zebra finches (*N*=65)

	Fat score	% NL	% Lipid
% NL	0.41		
% Lipid	0.32	0.46	
Adipose mass	0.55	0.27	0.29

non-significant (t_{55}=1.62, P=0.110). There was no effect of exercise on the mean carbon retention time in the NL fraction, despite a larger estimated difference between groups (Figs 1 and 2; exercised group: τ=22.4±7.1 days, control group: τ=17.8±5.2 days, t_{56}=0.569, P=0.571). There was no significant effect of sex on carbon turnover in either fraction. The measured $\delta^{13}C$ values of both NL and PL fractions at day 256 closely matched the $\delta^{13}C$ value of the lipids in the C_3 diet, in contrast to the poor match with the bulk diet and lipid-free diet (Fig. 2). Consequently, our estimated tissue–diet discrimination factors were close to zero for dietary lipids and 3–5‰ for bulk diet and lipid-free diet (Table 2).

Residual analyses

To help explain differences in the estimated variability of mean carbon retention time between NL and PL fractions, we tested for relationships between residual $\delta^{13}C$ values and both fat score and the proportion of NLs in the pectoral muscle, which we expected to be representative of the full-body lipid pool and muscle lipid pool sizes, respectively. Fat score, proportion of NLs in the pectoral muscle, lipid content of the pectoral muscle and adipose tissue mass were all positively correlated (Table 3), indicating that individual measures are representative of a more generalized response. There was a significant positive correlation between residual $\delta^{13}C$ values and fat score in the PL fraction (Fig. 3, r_{65}=0.30, P=0.01) but not the

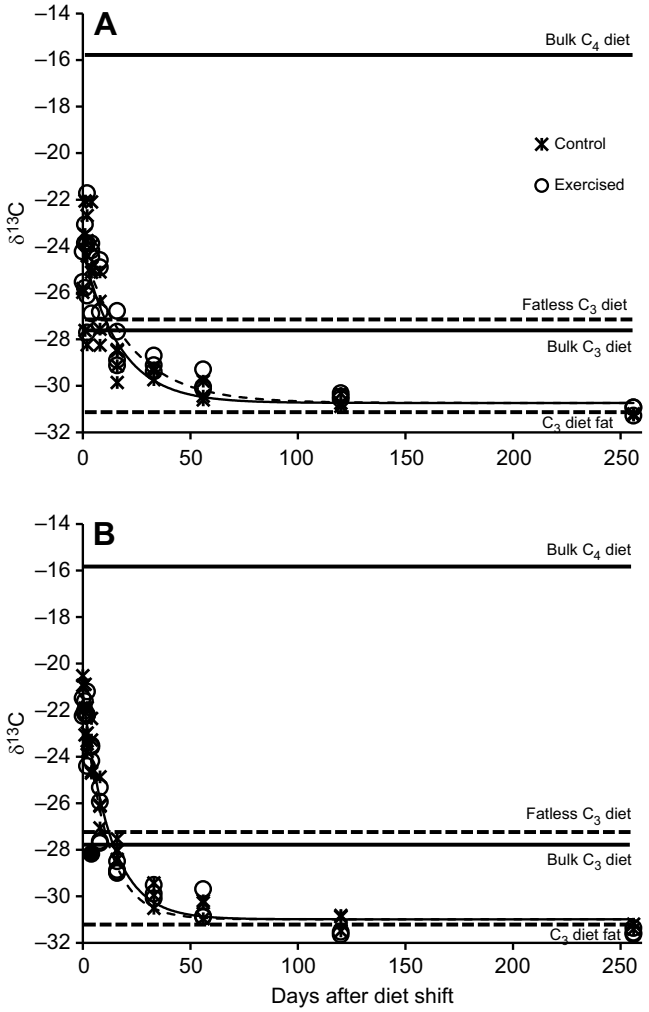

Fig. 2. Changes in $\delta^{13}C$ values of pectoral muscle neutral and polar lipids following a C_4 to C_3 diet shift in the zebra finch (*N*=65). (A) Neutral lipids (NLs); (B) polar lipids (PLs). Thin lines represent the exponential decay in $\delta^{13}C$ value for birds in the exercise (dashed) and control (solid) treatment groups. Solid bold horizontal lines represent the two bulk diets, while dashed bold horizontal lines represent fat and fat-extracted components of the C_3 diet. The statistical outlier in the polar fraction is designated by a filled circle.

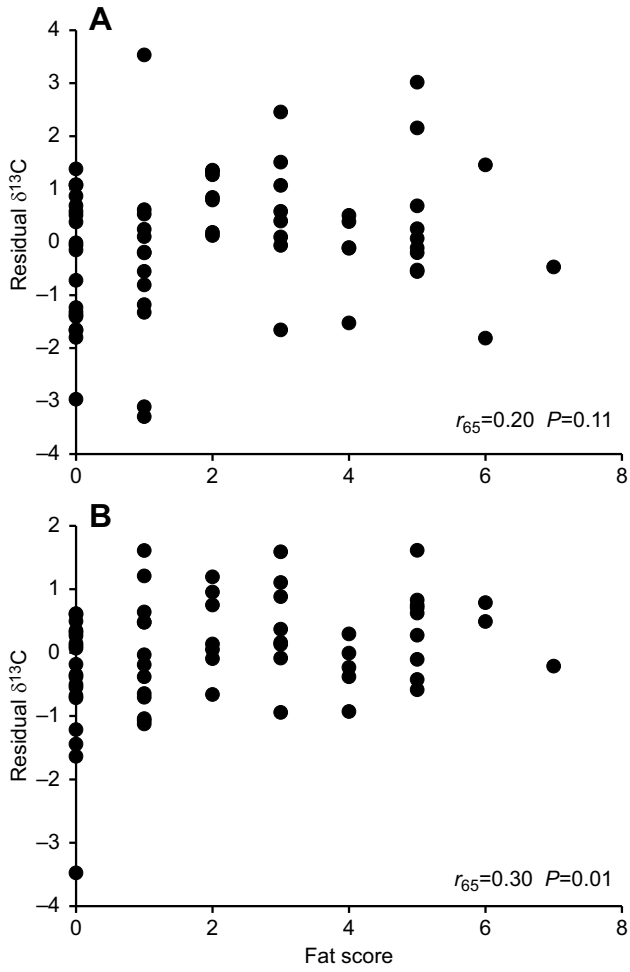

Fig. 3. Relationship between residual $\delta^{13}C$ value and fat score of pectoral muscle NLs and PLs in the zebra finch. (A) NLs; (B) PLs. Residuals were calculated from the best-fit non-linear regression model for each fraction and fat score was evaluated on a 0–7 scale, where 0 is no visible fat and 7 is complete coverage of the torso and abdomen with fat. Each point represents an individual bird. There was a significant correlation for polar fraction residuals (r_{65}=0.30, P=0.01) but not neutral fraction residuals.

NL fraction (r_{65}=0.20, P=0.11). Similarly, there was a significant positive correlation between residual PL fraction $\delta^{13}C$ values and the proportion of NLs in pectoral muscle (Fig. S1, r_{60}=0.30, P=0.02) but not residual NL fraction $\delta^{13}C$ values (r_{60}=−0.03, P=0.81). In contrast, there was no significant relationship between residual $\delta^{13}C$ values and any other measure of individual body condition, including body mass, pectoral muscle mass, liver mass or small intestine mass, for either lipid fraction (P>0.29 in all cases). However, the NL and PL fraction residuals were themselves significantly correlated (r_{63}=0.49, P<0.0001). We did not include exercise group as a covariate in any of our analyses involving residuals as there was no significant difference between groups in fat score or other metrics (P>0.09 in all cases).

DISCUSSION

We estimated mean retention times (τ) of approximately 19.5, 12.7 and 10.5 days for NL, control PL and exercised PL fractions, respectively (Fig. 1), which are equivalent to first-order kinetic rates ($1/\tau$) of 0.051, 0.079 and 0.093 day^{-1}, respectively. At these rates, 50% of lipids would be replaced in 13.5, 8.8 and 7.5 days for NL, control PL and exercised PL, respectively, and 95% of lipids would be replaced in 58.4, 38.0 and 32.4 days. These results are consistent with our expectation of faster turnover of the PL fraction than the NL fraction. These are, to our knowledge, the first estimates of lipid turnover in a songbird, the first in adult, non-growing birds, and the first to quantify the turnover of two biologically relevant classes of lipids. These estimates of turnover rate imply that in 2–3 weeks, small (10–20 g) songbirds have the potential to substantially change the composition of their intramuscular fuel stores and especially their lipid membranes, a time scale relevant to major life-history stages such as migration and reproduction. Our results also provide some support for the hypothesized effect of metabolic rate on lipid turnover, although this effect was contingent on a statistical outlier. Future studies with different exercise regimes (e.g. wind tunnel flight training; Hobson and Yohannes, 2007) and comparisons of turnover across tissues will be necessary to confirm the effect of metabolic rate and to relate overall tissue function (e.g. fuel storage in adipose tissue) to lipid turnover. Finally, our estimates of diet–tissue discrimination factors largely fall within expected ranges and support consistent mechanisms of discrimination across tissues and species.

Effects of flight training and metabolic rate

Although we hypothesized that flight training would be related to lipid turnover in both NL and PL fractions, we expected a greater effect on PLs because of a more localized effect of metabolic rate via oxidative damage (Boom et al., 1996; Samuni et al., 2000; Wongekkabut et al., 2007). We found faster turnover in the PL fraction of exercised zebra finches but not the NL fraction, suggesting that PLs of exercised birds are either replaced at higher rates than in sedentary birds or their PL fraction was increasing in size over time, more rapidly diluting the ^{13}C signature of the C_4 diet. It is unlikely that the PL fraction grew larger throughout the experiment as neither the lipid content of pectoral muscle samples nor the proportion of lipids in the polar fraction increased over the course of the study. There are, then, several mechanisms that could explain this apparent increase in the rate at which PLs are replaced. Flight is known to increase metabolic rate (Schmidt-Nielsen, 1997; Schmidt-Wellenburg et al., 2008), and increased metabolic rate is known to increase reactive species (Mataix et al., 1998; Speakman and Garratt, 2014). Reactive species can damage any molecule type, but they have been often linked to lipid peroxidation in birds (Costantini et al., 2007; Jenni-Eiermann et al., 2014; Skrip and McWilliams, 2016). Our flight training regimen likely increased damage to membrane phospholipids, necessitating their replacement and a consequent increase in turnover rate. Membrane phospholipids are also used as a reservoir for lipid-derived signaling molecules such as eicosanoids (Watkins, 1991; Zhou and Nilsson, 2001), which have been shown to be released at higher rates during exercise in rats (Chen et al., 1993). It is therefore possible that flight training increased the consumption of these lipids, again increasing turnover as they were replaced. We are unable to distinguish between these mechanisms without knowledge of how specific fatty acids are replaced in response to exercise. We also expected the consumption of triacylglycerols as fuel to contribute to an effect of flight training on NL turnover. The lack of any such pattern may suggest that the lipids used to fuel flight are primarily stored outside of muscle cells with little delay in supply that might force the use of more local NLs (Jenni and Jenni-Eiermann, 1998; Jenni-Eiermann et al., 2002).

The approximately 18% increase in turnover rate of the pectoral PLs with flight training was considerably smaller than the observed response of adipose tissue lipids to a thyromimetic hormone in juvenile chickens (an approximately 120% increase; Foglia et al., 1994). A number of factors may have contributed to this disparity. First, the supplementation of a thyromimetic hormone to an animal's diet is likely to affect metabolic rate more directly and for a greater amount of time than a daily 2 h flight training period (Sherman et al., 1997; Underwood et al., 1986). Second, growth is an important contributor to isotopic turnover (Hesslein et al., 1993; Martínez del Rio and Carleton, 2012) but was not part of our study of adult zebra finches, although this additional influence of growth was not incorporated into the model of turnover used by Foglia et al. (1994). Third, flight training has been linked to reductions in basal metabolic rate (Nudds and Bryant, 2001), so birds in our exercised group may have had reduced basal metabolic rates relative to control birds outside of flight training, thus reducing the magnitude of the difference in overall metabolic rate between these groups. Similarly, it is possible that exercised birds reduced activity outside of exercise training to compensate for the effects of flight training, although this is inconsistent with the results of a past study using this exercise protocol (Bauchinger et al., 2010). It is important to note that the observed effect of flight training was sensitive to the presence of a statistical outlier. There is, however, no evidence of any characteristic specific to that individual bird that could contribute to the abnormality of its $\delta^{13}C$ value and its residual is comparable in magnitude to that of neutral fraction measurements. We conclude that there is modest support for the relationship between flight-elevated metabolic rate and an increased turnover rate of the PL but not NL fractions.

Differences between lipid fractions

We found that pectoral muscle lipids turned over approximately 55–82% faster in the PL fraction than in the NL fraction, depending on exercise condition (Figs 1, 2). We also found considerably more variation in our estimate of turnover for the NL fraction (s.e.=3.2 days) than for the PL fraction (s.e.=1.4–1.7 days, depending on exercise condition), indicating that turnover was slower and more variable in the NL fraction. Given relatively constant starting and asymptotic $\delta^{13}C$ values – a reasonable assumption considering the consistency of diet–tissue discrimination factors across individuals and species (Hobson and Clark, 1992b; Lourenço et al., 2015; Mohan et al., 2016; Murray and Wolf, 2012) – much of the residual variation in $\delta^{13}C$ value at any given time point could be attributed to variation in turnover rates among individual birds. Thus, residual $\delta^{13}C$ values can be used as a

proxy for individual turnover rate, with more negative values indicating faster turnover and more positive values indicating slower turnover. In this context, the significant relationship between residual $\delta^{13}C$ values in the polar fraction and fat score suggests that turnover in the polar fraction is slower in fatter birds and faster in leaner birds. With a constant replacement rate, we would expect overall turnover to be slower in larger pools and faster in smaller pools (Martínez del Rio and Carleton, 2012; McCue, 2011), so this relationship can be explained by increases in the size of the lipid pool as birds fattened, including increases in fat score, the proportion of NLs in the pectoral muscle, lipid content of the pectoral muscle, and adipose tissue mass (Table 3). More broadly, we would expect the PL pool to be smaller than the NL pool as a result of the higher proportion of omega-3 and omega-6 fatty acids, which cannot be synthesized by vertebrates (Klasing, 1998; Lands, 2014; McCue et al., 2009; Stevens, 2004), and the localization necessary for phospholipid synthesis (Engelmann and Wiedmann, 2010; Kuchler et al., 1986; Rosenberger et al., 2009). Additionally, considerable variation has been observed among individuals in the activity of lipogenic enzymes in birds (Egeler et al., 2000; Rosebrough et al., 2007), contributing to greater inter-individual variation in the size of the NL pool. Together, these mechanisms could contribute to a larger and more variable lipid pool available to the NL fraction of pectoral muscle lipids, and thus explain the slower and more variable turnover of that fraction.

A second possibility is that the observed differences in NL and PL fractions are due to mechanistic differences in use. As described above, the structural role of phospholipids in cell and organelle membranes places them at risk of damage from RS, which may simply force PL molecules to be replaced at a faster rate than NL molecules are consumed and replaced as fuel. Polyunsaturated fatty acids are more vulnerable to oxidative damage than saturated or monounsaturated fatty acids (Hulbert, 2010; Skrip and McWilliams, 2016), so the PL fraction as a whole may be more responsive to oxidative damage as a result of its more polyunsaturated character (Guglielmo et al., 2002b; Klaiman et al., 2009; McCue et al., 2009). This hypothesis and the effect of pool size described above are not mutually exclusive; however, although it is reasonable to expect both to contribute, at present we believe that pool size is a more parsimonious explanation for the variability of turnover in different fractions. Future studies on the turnover of individual fatty acids with different properties (e.g. synthesizability, risk of damage, etc.) may be useful in sorting out the relative importance of these mechanisms.

Comparison to protein turnover

Turnover of the NL fraction was more similar than turnover of the PL fraction to published values of protein turnover in the pectoral muscle in zebra finches (τ=21±2.55 days; Bauchinger and McWilliams, 2009). However, neither fraction supported the use of a two-compartment model, in contrast with estimates of protein turnover in the pectoral muscle and many other tissues (Bauchinger et al., 2010; Carleton et al., 2008; Cloyed et al., 2015). The lack of support for two-compartment models in pectoral muscle lipids suggests that turnover of lipids is more continuous than for proteins, for which recycling may have increased importance because of the high proportion of essential amino acids (McCue, 2011; Stevens, 2004). Protein turnover has been largely linked to tissue-specific rates of protein synthesis and catabolism (Bauchinger and McWilliams, 2010; Carleton et al., 2008; Waterlow, 2006); therefore, the observed similarity in turnover between proteins and the NL fraction may be purely coincidental. In contrast to our study, no support has been found for an effect of exercise on non-lipid carbon turnover (Bauchinger et al., 2010; Hobson and Yohannes, 2007). Although the effect of exercise in our study was modest, it again highlights the differences in metabolism between proteins, carbohydrates and lipids.

Tissue–diet discrimination factors

Our estimates of tissue–diet discrimination factors were generally similar for the NL and PL fractions, with slightly larger discrimination in PLs (Table 2). Although most studies of tissue–diet discrimination have focused on lipid-free tissues, Podlesak and McWilliams (2007) estimated discrimination of approximately 3.2‰ between furcular fat in yellow-rumped warblers and bulk diet with a similar fat content (5%) to our C_3 diet. This value is similar to our estimate, and a previous study likewise found that discrimination was similar for furcular fat and pectoral muscle lipids (Podlesak and McWilliams, 2006), suggesting that physiologically similar mechanisms of fractionation are at work across tissues and species. Similarly, our estimates of discrimination between the non-lipid diet portion and pectoral lipids fall within the expected range of 4–8‰ (DeNiro and Epstein, 1977; Hammer et al., 1998), although our estimated lipid–lipid discrimination factors are smaller than estimates of whole lipids and individual fatty acids (Budge et al., 2011; Podlesak and McWilliams, 2007). This difference is small enough that it could be explained by variation in fractionation within that 4–8‰ range as carbohydrates are synthesized into lipids. Considering the relatively recent development of lipid carbon isotope signatures as a tool for diet reconstruction (Budge et al., 2008; Wang et al., 2014, 2015), these values may prove useful for future studies of songbird diet.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: W.C., S.R.M.; Methodology: W.C., C.C., S.R.M.; Validation: W.C.; Formal analysis: W.C.; Investigation: W.C., C.C., S.R.M.; Resources: S.R.M.; Data curation: W.C.; Writing - original draft: W.C.; Writing - review & editing: W.C., C.C., S.R.M.; Visualization: W.C.; Supervision: S.R.M.; Project administration: S.R.M.; Funding acquisition: S.R.M.

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Supplementary information

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Supplementary Figures

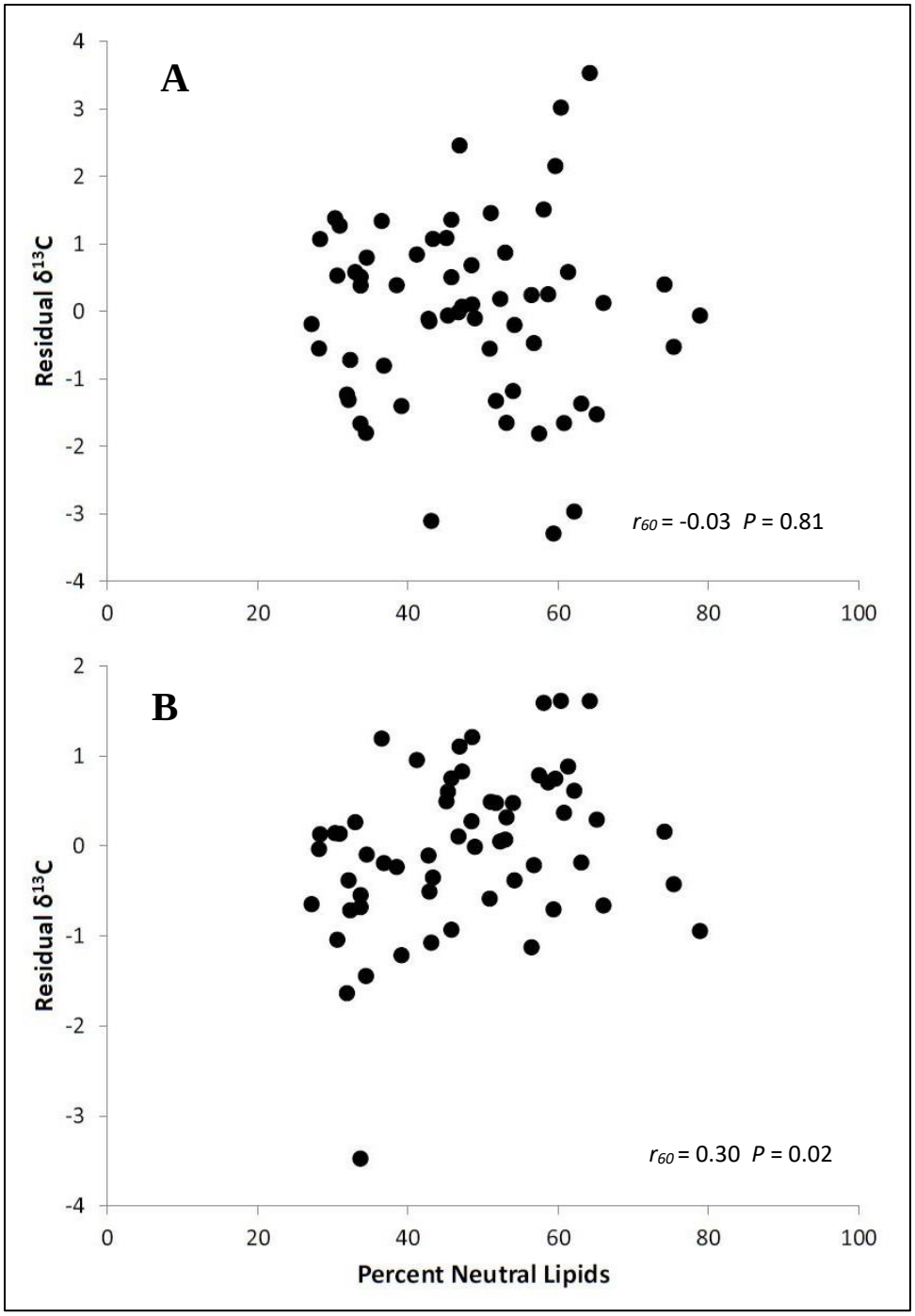

Fig. S1. The relationship between residual $\delta^{13}C$ value of Zebra Finch muscle lipids in the (A) neutral and (B) polar fractions and the proportion of sample lipids located in the neutral fraction. Residuals were calculated from the best-fit nonlinear regression model for each fraction and proportion in the neutral fraction was calculated as a percent of total lipid content by mass. Each point represents an individual bird. There was a significant correlation for polar fraction residuals (r_{60} = 0.30, P = 0.02) but not neutral fraction residuals.

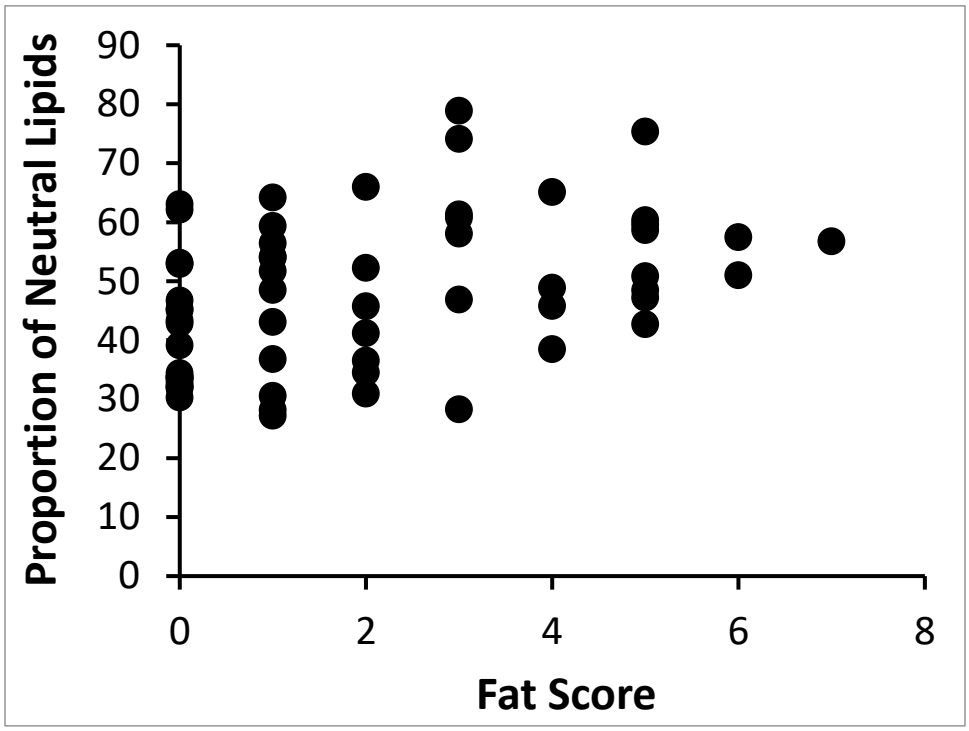

Fig. S2. There was a positive correlation between fat score and the proportion of total lipids extracted from Zebra Finch muscle samples located in the neutral fraction (r_{60} = 0.41, P = 0.01). Fat score was evaluated on a 0 to 7 scale, where 0 is no visible fat and 7 is complete coverage of the torso and abdomen with fat and proportion in the neutral fraction was calculated as a percent of total lipid content by mass. Each point represents an individual bird.

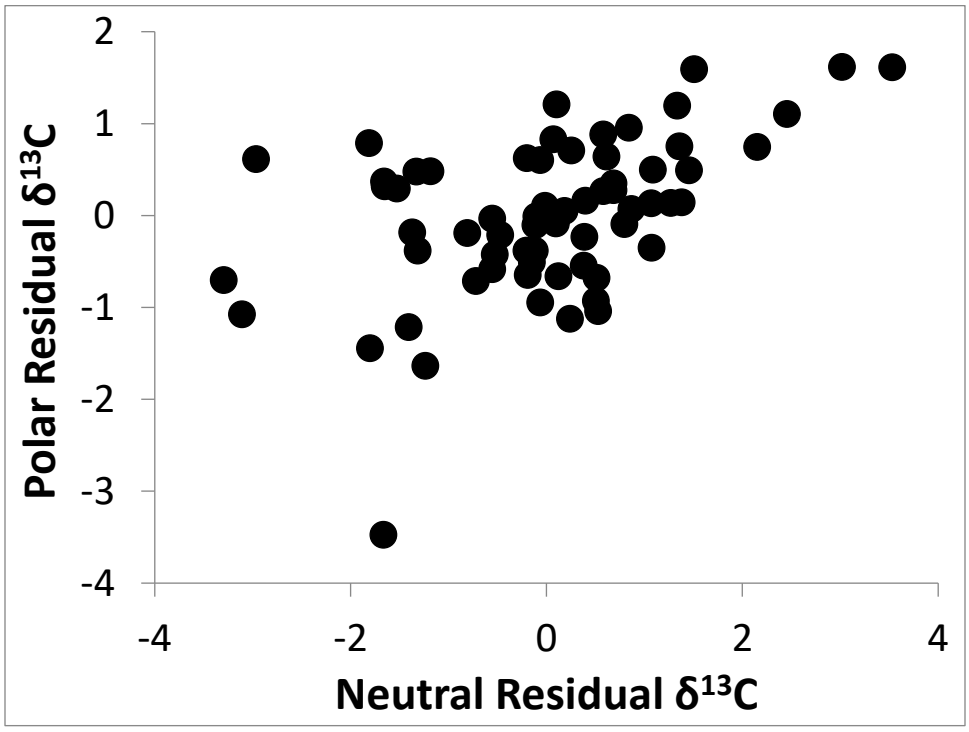

Fig. S3. The positive relationship between $\delta^{13}C$ values calculated from the best-fit nonlinear regression models for neutral and polar fractions of lipids extracted from Zebra Finch muscle samples ($r_{63} = 0.49$, $P < 0.0001$). Each point represents an individual bird.