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WHAT TO EAT AND WHEN: THE CONSEQUENCES OF
POLYUNSATURATED FAT CONSUMPTION AND PACE OF CHANGES TO
BODY LIPID COMPOSITION IN SONGBIRDS

BY

WALES ARAN CARTER

A DISSERTATION SUBMITTED IN PARTIAL FULFILLMENT OF THE
REQUIREMENTS FOR THE DEGREE OF

DOCTOR OF PHILOSOPHY

IN

BIOLOGICAL AND ENVIRONMENTAL SCIENCES

UNIVERSITY OF RHODE ISLAND

2019

DOCTOR OF PHILOSOPHY DISSERTATION
OF
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2019

ABSTRACT

Diet mediates environmental influence on organismal performance, but diets must be broken down into their constituent compounds and integrated into organismal tissues before they can exert such influence. Thus, a full account of the relationship between diet and organismal performance requires descriptions of intermediate effects on body composition and tissue function, as well as the dynamics of composition and function over time. The work described in this dissertation investigates a case study of diet and organismal performance in songbirds: dietary polyunsaturated fats have been demonstrated to influence the metabolic performance of songbirds, but the influence of individual fatty acid compounds, the mechanisms mediating dietary influence, and the time scales on which dietary fatty acids are integrated into songbird tissues are largely unknown. Using diet manipulations, respirometry, and wind-tunnel flight training, we tested the influence of dietary linoleic acid (18:2n6), a fatty acid implicated in previous studies, on tissue fatty acid composition and whole-animal metabolic performance in European Starlings (*Sturnus vulgaris*; Manuscript I), as well as its effect on tissue function, measured in terms of the activity and expression of fatty acid transporters and catabolic enzymes (Manuscript II). In that same experiment we also considered the effects of dietary antioxidants and training, whose influence was hypothesized to interact with that of linoleic acid. To complement those studies, we also used a diet switch experiment and stable isotope analysis to estimate the turnover rates of tissue lipid compartments (Manuscript III) and individual fatty acid compounds (Manuscript IV) in Zebra Finch (*Taeniopygia guttata*) muscle in order to investigate the pace of changes in tissue composition and

the mechanisms governing the turnover of lipids in avian tissues. Finally, in Manuscript V we conduct a literature review and discuss the importance of knowing the pace of changes in tissue composition and the speed of dietary influence for studying the ecology of a wide range of species.

We found that changes in dietary linoleic acid are enough to substantially change the fatty acid composition of muscle and to influence resting, maximal, and sustained metabolic rates in starlings, although this influence was more context dependent than has been found in previous studies. Similarly, dietary linoleic acid, antioxidants, and training did influence the activity of oxidative enzymes in starling muscle, but the effects of linoleic acid depended on the overall protein levels in the tissue, which were, in turn, variable over time. Together, these results suggest that the influence of dietary linoleic acid largely depends on its properties as a signaling molecule and that it could be used by songbirds to direct and control preparations for migration. We also found that lipid membranes turn over more quickly than triglyceride stores in zebra finch muscle and there is substantial variation in turnover rates among fatty acids, with linoleic acid and palmitic acid (16:0) having fast turnover and long chain polyunsaturated fatty acids having slow turnover. These results demonstrate that the lipid composition of songbird tissues, and especially cell and organelle membranes are highly dynamic and can respond to changes in diet within days. Furthermore, these results and those of similar studies provide information critical for the assessment of nutrition and ecology of wild animals.

ACKNOWLEDGMENTS

This work represents the culmination of an extended process that would not be possible without the contributions and influence of an inordinately large number of people. Below, I would like to recognize and thank those most directly connected.

First and foremost, I would like to thank Dr. Scott R. McWilliams, my advisor and mentor. Not only did he provide invaluable guidance and support for the scientific direction of these projects, but he was also an essential resource for the entire process of navigating graduate school and my professional development. I have enjoyed numerous opportunities and connections that would not have been possible with him. I cannot imagine going through this process without him and I'm sure that I will discover new ways that he has influenced me throughout my life.

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Skip, who laid the foundations for all subsequent ZB work, Adam Smith, Dustin Meatty, Clay Graham, Dr. Juliet Lamb, Abby Frawley, and Erin Harrington.

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Naturally, I must thank my parents, not only for my existence, but for their support and upbringing, which laid the groundwork for me to take this path in life.

Finally, and perhaps most importantly, I must thank the 108 starlings and 65 zebra finches who (unwittingly) gave their lives to make this work possible. Small consolation though it may be, I would not want them to think that their sacrifice went unnoticed and unappreciated.

PREFACE

This dissertation is written in manuscript format, with individual chapters prepared as manuscripts for publication in peer-reviewed journals. Manuscript I was prepared for and submitted to *Oecologia*, where it is currently under review. Manuscript II is prepared for submission to the *Journal of Comparative Physiology B: Biochemical, Systems, and Environmental Physiology*. Manuscript III was prepared for and submitted for publication to the *Journal of Experimental Biology*. It was accepted for publication in February 2018. Manuscript IV was prepared for and submitted for publication to *Physiological and Biochemical Zoology*. It was accepted for publication in November 2018. Manuscript V was prepared for and submitted for publication to *Diversity*. It was accepted for publication in May 2019.

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MANUSCRIPT I:

The effects of dietary linoleic acid and hydrophilic antioxidants on basal, peak, and
sustained metabolism in European Starlings

by

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Prepared for and submitted to *Oecologia* in August 2019, currently in review

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Abstract

Dietary micronutrients have the ability to strongly influence animal physiology and ecology. For songbirds, dietary polyunsaturated fatty acids (PUFAs) and antioxidants are hypothesized to be particularly important micronutrients because of their influence on an individual's capacity for aerobic metabolism and recovery from extended bouts of exercise. However, the influence of specific fatty acids and hydrophilic antioxidants on whole-animal performance remain largely untested. We used diet manipulations to directly test the effects of dietary PUFA, specifically linoleic acid (18:2n6), and anthocyanins, a hydrophilic antioxidant, on basal metabolic rate (BMR), peak metabolic rate (PMR), and rates of fat catabolism, lean catabolism, and energy expenditure during sustained flight in a wind tunnel in European starlings (*Sturnus vulgaris*). BMR, PMR, energy expenditure, and fat metabolism decreased and lean catabolism increased over the course of the experiment in birds fed a high (32%) 18:2n6 diet, while birds fed a low (13%) 18:2n6 diet exhibited the reverse pattern. Additionally, energy expenditure, fat catabolism, and flight duration were all subject to diet-specific effects of body fat content. Dietary antioxidants and diet-related differences in tissue fatty acid composition were not directly related to any measure of whole-animal performance. Together, these results suggest that the effect of dietary 18:2n6 on performance was most likely the result of molecular signaling properties. This implies that dietary PUFA influence the energetic capabilities of songbirds, and could strongly influence songbird ecology, given their availability in terrestrial systems.

Keywords

Fatty acids, antioxidants, BMR, PMR, sustained metabolism, songbirds, flight training

Introduction

The success of individual animals depends on their ability to acquire diets with sufficient resources for maintenance, activity, and reproduction, which in turn shapes ecological niches and interactions among species. Besides energy and macronutrient content, the adequacy of animal diets often depends on their micronutrient composition, as demonstrated, for example, by the influence of sodium and calcium on the habitat selection, movements, and population dynamics of mammals and birds (Aumann and Emlen 1965; Belovsky and Jordan 1981; Wilkin et al. 2009). Dietary fatty acids include essential micronutrients, polyunsaturated fatty acids (PUFA), with demonstrated effects on ecologically-relevant performance of a wide range of taxa. For example, dietary PUFA affects active and resting metabolic rates in songbirds and humans (van Marken Lichtenbelt et al. 1997; Pierce et al. 2005; Twining et al. 2016), mitochondrial metabolism in ground squirrels (Gerson et al. 2008), and aerobic endurance and efficiency in songbirds (McWilliams and Pierce 2006), rats and hares (Helge et al. 1998; Ruf et al. 2006) and salmon (McKenzie and Higgs 1998). Results such as these suggest that 1) the fatty acids that are important micronutrients for many wild vertebrates are primarily long-chain polyunsaturated, likely due to their unique chemical properties and limited synthesis by vertebrates (Klasing 1998; Twining et al. 2018); 2) dietary fatty acids seem to primarily influence

the regulation and scope of energy metabolism (Price 2010; Pierce and McWilliams 2014); 3) the effects of dietary fatty acids have particular relevance for birds during migration, possibly due to the high metabolic demands of flight (Guglielmo 2010; Martinez del Rio and McWilliams 2016). However, the effects of dietary fatty acids on different measures of performance and different species of migratory birds have been equivocal (e.g. Pierce et al. 2005; Price and Guglielmo 2009), suggesting a need for more refined tests of the influence of individual fatty acids.

Dietary antioxidants are also important micronutrients for birds, in general (Klasing 1998), and for birds in migration, specifically (Alan et al. 2013; Bolser et al. 2013). Functionally, dietary antioxidants act to reduce oxidative damage (Catoni et al. 2008a; Beaulieu and Schaefer 2013; Skrip and McWilliams 2016), as well as influence immune function (Catoni et al. 2008b; Marri and Richner 2015), investment in reproduction (Royle et al. 2003; Skrip et al. 2016), reproductive hormones and behavior (Carbeck et al. 2018), and ability to meet the oxidative costs of flight (Larcombe et al. 2008; Skrip et al. 2016). However, as with fatty acids, results have differed among studies that used different measures of performance and that supplemented diets with different antioxidants, with hydrophilic antioxidants being largely untested (Cooper-Mullin and McWilliams 2016).

Dietary fatty acids and antioxidants may also interact during their metabolism and synergistically affect animal performance. High-PUFA diets and subsequently enhanced metabolism may be beneficial when energy demands are elevated by long bouts of flight (Wikelski et al. 2003; Dingle 2014), as when minimizing time on migration (Alerstam and Hedenstrom 1998). However, elevated metabolism also

generally involves the increased production of pro-oxidants (Mataix et al. 1998; Larcombe et al. 2008; Jenni-Eiermann et al. 2014) and the storage of PUFAs increases the risk of lipid peroxidation (Hulbert 2010; Skrip et al. 2015; Skrip and McWilliams 2016). Thus, songbirds must balance the need to meet high metabolic demands with the risk of oxidative damage, and this tradeoff may be mitigated with the intake of dietary antioxidants. Additionally, migratory songbirds undergo a range of physiological changes in preparation for migration including the upregulation of lipid transporters, oxidative enzymes, and hormones (McFarlan et al. 2009; Price et al. 2011; Corder et al. 2016), which could interact with dietary fatty acids or antioxidants to produce changes in performance over time.

We experimentally tested how dietary linoleic acid (18:2n6), a leading candidate to mediate the effects of dietary fatty acid composition on metabolism in songbirds (Pierce et al. 2005; Price and Guglielmo 2009; Pierce and McWilliams 2014), and the dietary anthocyanins, hydrophilic antioxidants common in the diets of wild songbirds (Bolser et al. 2013; Cooper-Mullin and McWilliams 2016) interact to affect exercise performance of European starlings (*Sturnus vulgaris*). We measured performance in terms of metabolic rates and tissue catabolism during sustained flight in a wind tunnel to allow us to assess the effect of diet on basal, peak, and sustained metabolism. This study provides the first test of the hypothesis that performance of songbirds during sustained flight is influenced by both dietary fatty acids and water-soluble antioxidants.

Methods

Animal Care and Experimental Diets

This experiment took place from August to December, 2015 at the Advanced Facility for Avian Research (AFAR) at the University of Western Ontario (UWO) in London, ON, CA, and was covered by animal care protocols for UWO (2010-216) and the University of Rhode Island (AN11-12-009). Between 19-23 August 2015 we captured 108 hatch-year European starlings (*Sturnus vulgaris*) at a dairy farm approximately 20 km north of UWO in Middlesex Center, ON and immediately transported birds to AFAR where they were housed in four large indoor aviaries (two 2.4m x 3.7m x 3.1m and two 2.4m x 2.3m x 3.5m). Migratory behavior has been inferred from band returns in this population (Cabe 1993), but may not extend to the majority of individuals. On August 24th we measured the body mass, morphometrics, and molt score (0 – 75; Ginn and Melville 1983) of each bird and randomly resorted the birds into the four aviaries such that each aviary housed a group of birds with roughly equal body mass and molt score distributions. Aviaries were kept at 21°C and on a natural light cycle for London, ON (14.5 hrs light : 9.5 hrs dark at capture). Each week we weighed, measured molt, and otherwise inspected birds to monitor their health.

Starting at capture, birds in each aviary had *ad libitum* access to water and one of two isocaloric, semi-synthetic diets that differed only in fatty acid (FA) composition (Table 1). Diets were either high (32%) or low (13%) in 18:2n6. We lightly supplemented the diet with dried mealworms for three weeks post-capture to facilitate the birds' transition from natural to experimental diets in captivity. Starting on September 1st we supplemented the diets of birds in one 32% and one 13% 18:2n6

aviary with dried elderberry powder (Artemis International, Fort Wayne, IN) while making the diets as a source of hydrophilic antioxidants (AOX). The vitamin mix added to all diets provided a low level of lipophilic dietary antioxidants (7.6mg/g dry diet α -tocopherol). Thus, after 1 September the experimental design was a 2 x 2 factorial with four groups: 32% 18:2n6 high AOX ($N = 28$), 32% 18:2n6 low AOX ($N = 27$), 13% 18:2n6 high AOX ($N = 27$), and 13% 18:2n6 low AOX ($N = 26$).

Cohort Assignment and Metabolic Measurements

On September 21st we fixed the light schedule at 11:13 L:D and assigned birds in each diet group to one of five cohorts ($N = 5$ -6 per cohort) in descending order of molt score (i.e., from most to least advanced in molt) that corresponded to the order in which they would be flight trained in the wind tunnel. The resulting twenty cohorts were then ordered by the average molt score of birds within each cohort. Starting on September 23rd, and continuing every three days thereafter, we removed the individuals of the appropriate cohort from their aviary, and then weighed, blood sampled, and measured the body composition (fat and lean mass) of each individual with a quantitative magnetic resonance (QMR; Echo Medical Systems, Houston, TX) instrument calibrated daily with a canola oil standard (Guglielmo et al. 2011). Three of the five individuals in each cohort were then randomly assigned to the flight-training group and two were assigned to the untrained control group. Birds were then moved to individual 0.6m x 0.5m x 0.5m cages with *ad libitum* access to food and water for two days (day -9 and -8 relative to the start of flight training for a given cohort, see below), and on day -7 we measured birds' food intake.

Starting at 8:00 PM on the night of day -6 we used flow-through respirometry to measure the pre-training basal metabolic rate (BMR) of the three birds assigned to the flight-training and one control bird. Dried air flowed through four individual chambers housed in an incubator set at 27°C (Wiersma et al. 2005) and the gas composition of effluent air was measured in a rotation of the background source and individual chambers. Flow rate and partial pressures of oxygen, carbon dioxide, and water vapor were measured with a Sable Systems (Las Vegas, NV) Flowbar-8 Flow Controller, FC-1b O₂ Analyzer, CA-2A CO₂ Analyzer, and RH-300 Water Vapor Analyzer, respectively. Since our questions involved relative comparisons of diet groups, we present BMR as the lowest instantaneous oxygen consumption averaged over a five-minute period, corrected for CO₂ and water vapor concentrations. This period occurred more than six hours after the start of the trial for all birds, supporting the assumption that the birds were post-absorptive.

The following morning (day -5), we measured the peak metabolic rate (PMR) of the same four birds in a flight-hover wheel similar to those used by Pierce et al. (2005) and Price and Guglielmo (2009) using the same gas analyzers as above and an MFC-2 flow controller. The internal width and diameter of the wheel were both 30 cm, and the flow rate of air was 5 L/min. For each trial, we measured the baseline gas concentrations for two minutes before the bird was weighed, introduced to the chamber, and given five minutes to become acclimated. After the acclimation period, the wheel was spun slowly (~30 RPM) for two minutes and then ramped up to a speed where the bird's wings were in constant motion (~60 – 90 RPM). This pace was held until the bird's oxygen consumption plateaued or declined for at least two

minutes, after which the bird was removed and gas concentrations were allowed to return to baseline. We calculated PMR as the highest instantaneous oxygen consumption averaged over a one-minute period, which occurred 15 – 30 minutes after the start of the PMR measurements for all birds. Following PMR measurements, flight-training birds were moved to a 0.8m x 1.5m x 2m flight aviary and control birds were returned to their initial aviaries. Control birds did not differ from exercise training birds at the time of metabolic rate measurements and were not used in subsequent tests of exercise performance.

Flight Training and Exercise Performance

In order to assess the effect of diet on sustained exercise performance, we put the three flight-training birds in each cohort through four days of pre-training and a fifteen-day wind tunnel flight-training regimen, which has demonstrated success at eliciting long-duration flights (Engel et al. 2006). Pretraining (day -4 to day -1) included training birds to fly between their flight cage and the wind tunnel and twenty minutes of acclimation time per day inside the wind tunnel with a perch available and at progressively increasing wind speeds (0 – 12 m/s). Flight-training consisted of a one hour fast and then continuous flight at 12m/s wind speed, 15°C, and 70% humidity for increasing durations over the course of the training regimen: day 1, 20 min; day 2, 20 min; day 3, 20 min; day 4, 20 min; day 5, 30 min; day 6, 30 min; day 7, 60 min; day 8, 90 min; day 9, 30 min; day 10, 120 min; day 11, 180 min; day 13, 60 min; day 14, 30 min. Day 12 was a rest day, during which we returned birds to individual cages and measured food intake. At 8:00 AM on day 14 we collected a

blood sample from each bird for measurements of pre-flight levels of plasma metabolites. Flight training concluded on day 15 with a flight that started at 8:00 AM and lasted as long as birds would voluntarily fly (maximum of 360 minutes). Body composition was measured with the QMR within 20 min of starting their longest flight. Immediately after the flight, we took a second blood sample for measurements of post-flight plasma metabolite levels and again measured the body composition of each bird with the QMR.

We used several metrics to assess the effect of diet on sustained exercise performance during long flights on day 15, including flight duration, the rates of fat and lean tissue catabolism during flight, and the rate of energy expenditure during the flight. Rates of fat and lean tissue catabolism were estimated by dividing the difference between pre- and post-flight fat and lean masses, obtained from the QMR, by flight duration. The rate of energy expenditure was estimated by multiplying the mass of fat and lean tissue lost during flight by their respective energy densities (39.7 kJ/g and 17.8 kJ/g; Schmidt-Nielsen 1997), adding them, and dividing by flight duration. All raw fat and lean mass measurements were corrected to dry masses following Guglielmo et al. (2011) before calculations.

Tissue Fatty Acid Composition

Untrained control birds and flight-training birds from a given cohort were euthanized and tissue samples taken on days 16 and 17, respectively, as part of a larger study on the effects of diet and training on exercise physiology. The sex of each bird was confirmed during dissection. To verify the effect of diet on tissue fatty

acid composition we analyzed the composition of pectoral muscle lipid membranes and intracellular fat droplets extracted using a modified Folch method (Folch et al. 1957; Guglielmo et al. 2002). Briefly, we homogenized approximately 200 mg of wet tissue in 6mL of 2:1 chloroform:methanol, centrifuged at 3000rpm for 15 minutes, separated aqueous solutes by rinsing with 0.25% KCl, and transferred the organic phase to a glass vial, where it was dried under N₂ and resuspended in chloroform. We separated whole lipid samples into lipid droplet, non-esterified fatty acid (NEFA), and membrane fractions in solid phase extraction columns (Supelco, LC-NH₂, 1mL aminopropyl bonding) with elutions of 2:1 chloroform:isopropanol, 49:1 isopropyl ether:acetic acid, and methanol. We collected lipid droplet and membrane fractions and then esterified them into fatty acid methyl esters (FAMES) by heating at 90°C for 2hrs in 1M acetyl chloride in methanol. Duplicate 1μl aliquots of sample FAMES (1mg/ml in dichloromethane) were injected into a Shimadzu Scientific Instruments QP2010S GC-MS linked to a 2010 FID (Shimadzu Scientific Instruments, Kyoto, Japan) 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 USA) 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).

Plasma Metabolites

To confirm the fuels used during flight (Jenni-Eiermann et al. 2002; Smith and McWilliams 2009; Skrip et al. 2015), we measured the concentrations of

triglycerides, uric acid, and β -hydroxybutyrate in the pre- and post-flight plasma samples taken on day 15. Blood samples were collected in capillary tubes, centrifuged at 5000 rpm for 10 minutes, and plasma was separated and stored at -80°C until analysis. We assayed metabolite concentrations on 96-well plates with a Biotek Synergy HTX plate reader (Biotek Instruments, Winooski, VT) using commercial kits adapted for use with small volumes. Triglycerides and uric acid were diluted 1:1 with 0.9% saline and measured using an absorbance endpoint assays (Sigma-Aldrich, St. Louis, MO and TECO Diagnostics, Anaheim, CA, respectively). β -hydroxybutyrate was diluted 1:4 with tris buffer (pH 8.5) and measured using an absorbance endpoint assay (Cayman Chemical, Ann Arbor, MI). All assays were run in duplicate and concentrations are presented mmol/L.

Statistical Analyses

All statistical analyses were completed in R (v3.3.2; R Core Team, Vienna, Austria). We analyzed metabolic data ($N = 76$) using linear models that tested the effects of dietary FA, dietary AOX, date, and sex and we included body mass as a covariate for both BMR and PMR and time of day as a covariate for PMR. Similarly, we used linear models with the above effects and pre-flight fat mass to describe the exercise performance of birds who completed flights longer than two hours ($N = 43$), the minimum time necessary for reliable changes in body composition. We also tested for effects of diet and date on body composition (pre-flight fat and lean masses). We used a Hotelling's T^2 test to compare overall fatty acid composition between 32% and 13% 18:2n6 groups and tested for correlations between the composition of 18:2n6 and

metabolic rates and exercise performance. Finally, we compared log transformed pre- and post-flight plasma metabolite levels with linear mixed models including time (pre- or post-flight), date, and diet as fixed effects and individual as a random effect. Degrees of freedom for linear mixed models were obtained using a Satterthwaite approximation.

Results

Metabolic Rates

We found a consistent relationship between metabolic rates, date, and dietary FA composition (figure 1, table 2): the metabolic rates of birds fed the 32% diet started high and decreased over time, while those of birds fed the 13% diet started low and increased over time (Diet x Date interaction: BMR: $T_{69} = 3.228$, $P = 0.002$; PMR: $T_{68} = 2.118$, $P = 0.038$). PMR significantly increased with body mass ($T_{68} = 2.915$, $P = 0.005$), whereas BMR did not significantly change with body mass ($T_{69} = 1.049$, $P = 0.298$). Time of day, dietary AOX, and sex were not significantly related to metabolic rates (Table 2). BMR and PMR were not correlated with each other ($T_{72} = 0.501$, $P = 0.618$).

Exercise Performance

Pre-flight fat mass did not significantly change over time ($T_{39} = -0.095$, $P = 0.925$) or differ between diet groups ($T_{39} = -1.067$, $P = 0.292$). There was a trend for pre-flight lean mass to decrease over the course of the experiment ($T_{39} = -2.019$, $P = 0.051$), but lean mass did not differ between diet groups ($T_{39} = -0.588$, $P = 0.560$). As with the

metabolic rates, we found significant relationships between dietary FA composition, date, and our measures of exercise performance (table 3). For flight duration, there was a significant three-way interaction between dietary FA composition, date and pre-flight fat mass ($T_{32} = -2.084$, $P = 0.045$): flight duration in the 32% group was stable over time and increased with pre-flight fat mass, whereas flight duration in the 13% group increased with the product of date and pre-flight fat mass (figure 2) resulting in birds with higher fat loads increasing their flight duration over time.

The rate of fat catabolism was subject to significant interactions between dietary FA composition and pre-flight fat mass ($T_{34} = 2.885$, $P = 0.006$) and between dietary FA composition and date ($T_{34} = -3.812$, $P < 0.001$): fat catabolism in the 32% group decreased over time and increased with fat mass, but was relatively stable across time and fat masses in the 13% group (figure 3 a, b). The rate of lean catabolism decreased for all birds as fat mass increased ($T_{35} = -2.187$, $P = 0.036$) and, similar to fat catabolism, was influenced by an interaction between dietary FA composition and date ($T_{35} = 2.289$, $P = 0.028$): lean catabolism increased over time in the 32% group but was relatively stable over time in the 13% group (figure 3 c, d).

Energy expenditure exhibited a similar pattern to fat catabolism with significant interactions between dietary FA composition and pre-flight fat mass ($T_{34} = 2.386$, $P = 0.023$) and between dietary FA composition and date ($T_{34} = -2.458$, $P = 0.019$): the rate of energy expenditure increased with fat mass and decreased over time in the 32% group but was relatively stable across fat masses and dates in the 13% group (figure 4). Body mass was a significant covariate in models of energy expenditure ($T_{34} = 2.950$, $P = 0.006$) and fat catabolism ($T_{34} = 3.730$, $P < 0.001$), but

not flight duration ($T_{32} = 0.694$, $P = 0.493$) or lean catabolism ($T_{35} = -1.01$, $P = 0.320$). Neither sex nor dietary AOX were significantly related to exercise performance (Table 3). Fat catabolism was positively correlated with energy expenditure ($\rho = 0.932$, $T_{41} = 16.51$, $P < 0.001$) and lean catabolism was negatively correlated with flight duration ($\rho = -0.507$, $T_{41} = -3.764$, $P = 0.008$) and fat catabolism ($\rho = -0.342$, $T_{41} = -2.329$, $P = 0.025$), but none of the measures of exercise performance were correlated with BMR or PMR (all $P > 0.336$).

Tissue Fatty Acid Composition

As expected given the experimental diets, pectoralis muscle fatty acid profiles of both lipid droplets (Neutral fraction; $T^2_{5,72} = 46.002$, $P < 0.0001$) and membranes (Polar fraction; $T^2_{6,76} = 38.177$, $P < 0.0001$) significantly differed between diet FA groups (figure 5, table 4). Lipid droplets were primarily composed of palmitic acid (16:0), oleic acid (18:1n9), and 18:2n6. Concentrations of 16:0 were higher in the 13% 18:2n6 group ($T_{72} = 2.396$, $P = 0.0192$) whereas 18:2n6 was higher in the 32% 18:2n6 group ($T_{73} = 11.643$, $P < 0.0001$), but both had higher concentrations when birds were trained (16:0, $T_{72} = -4.854$, $P < 0.0001$; 18:2n8, $T_{73} = -2.366$, $P = 0.0206$; figure 6). There was also an increase in 16:0 over time that was specific to the 13% 18:2n6 group ($T_{72} = -3.202$, $P = 0.002$; figure 6). In contrast, membranes contained appreciable concentrations of 16:0, 18:0, 18:1n9, 18:2n6, arachidonic acid (20:4n6), and docosahexaenoic acid (22:6n3). Concentrations of both 16:0 ($T_{78} = -6.756$, $P < 0.0001$) and 18:1n9 ($T_{78} = -7.7471$, $P < 0.0001$) were higher in the 13% 18:2n6 group, whereas 18:0 ($T_{78} = 3.530$, $P = 0.0007$) and 18:2n6 ($T_{78} = 9.075$, $P < 0.0001$) were

higher in the 32% 18:2n6 group. As with lipid droplets, 16:0 ($T_{78} = -2.335$, $P = 0.0221$) and 18:2n6 ($T_{78} = -3.106$, $P = 0.0026$) were more concentrated when birds were trained, but 18:0 ($T_{78} = 2.170$, $P = 0.033$) and 22:6n3 ($T_{78} = 5.095$, $P < 0.0001$) were higher when birds were untrained (figure 7). Meanwhile, 18:1n9 had lower concentrations when birds were supplemented with anthocyanins ($T_{78} = 2.153$, $P = 0.0344$). Fatty acid concentrations were stable over time in membranes except for subtle decreases in 18:0 ($T_{78} = -2.470$, $P = 0.0157$), and increases in 18:2n6 ($T_{78} = 2.516$, $P = 0.0139$). The only correlations between fatty acid composition and whole-animal performance both involved lipid droplet 16:0, which was positively related to flight duration ($\rho = 0.407$, $T_{38} = 2.748$, $P = 0.0091$) and negatively related to lean catabolism ($\rho = -0.377$, $T_{38} = -2.509$, $P = 0.0165$). However, both of these correlations were driven by differences in 16:0 content between diet groups.

Plasma Metabolites

Log-transformed plasma β -hydroxybutyrate concentrations were significantly elevated in post-flight samples relative to pre-flight samples (figure 8; $T_{49} = 10.475$, $P < 0.001$). Conversely, log-transformed triglyceride concentrations were significantly depleted in post-flight samples (figure 8; $T_{95} = -12.405$, $P < 0.001$). Log-transformed uric acid concentrations were elevated in post-flight samples ($T_{46.55} = 11.326$, $P < 0.001$) and also increased over the course of the study ($T_{45.41} = 2.134$, $P = 0.038$). Although the variance associated with individual was only significant for β -hydroxybutyrate (BUTY: $X^2_1 = 25.05$, $P < 0.0001$; TRIG: $X^2_1 = 0.01$, $P = 0.99$; UA: $X^2_1 = 2.74$, $P < 0.098$), removal of the random effect from the other models did not

qualitatively change these results. Post-flight uric acid concentrations were positively related to flight duration ($\rho = 0.327$, $T_{48} = 2.400$, $P = 0.020$). Dietary FA and AOX composition had no effect on any metabolite concentrations.

Discussion

Dietary Fatty Acids and Whole-Animals Performance

In contrast to previous studies with similar, but not identical, diet manipulations (Pierce and McWilliams 2005; McWilliams and Pierce 2006; Price and Guglielmo 2009), our diet manipulation did not produce static differences in performance between diet groups. Instead, we found similar patterns of diet-specific change over the course of the experiment in most of our measures, with birds fed the 32% 18:2n6 diet experiencing decreases in BMR, PMR, rate of fat catabolism, and rate of energy expenditure over time and birds fed the 13% 18:2n6 diet increasing in those measures over time. The two exceptions were the rate of lean catabolism, which exhibited a mirrored pattern of decrease in the 13% group and increase in the 32% group, and flight duration, which didn't change over time in the 32% group but increased with both time and fat load in the 13% group. Additionally, flight duration, fat catabolism, and energy expenditure all displayed diet-specific relationships with pre-flight fat load. Thus, a complete explanation of these results will both link dietary FA composition with performance and describe how this relationship changes with time and fat load.

One explanation is that PUFAs generally have higher mobilization and diffusion rates than MUFAs or SFAs of similar size (Raclot 2003; Price et al. 2008).

Thus, fat stores with higher concentrations of PUFAs could be transported to the mitochondria at higher rates, resulting in higher metabolic rates and rates of fat catabolism. This is consistent with the higher concentrations of 18:2n6 in muscle lipid droplets, basal and peak metabolic rates, and rates of fat catabolism and energy expenditure in the 32% group early in the experiment. Similarly, higher concentrations of 18:2n6 could result in greater total amounts of fat mobilized in the course of a flight, explaining the diet-specific relationship between fat catabolism and fat load. However, the concentration of 18:2n6 in lipid droplets remained stable throughout the experiment and so cannot explain the diet-specific changes in performance over time. Lipid droplet 18:2n6 concentration was also not correlated with any measure of performance, as we would expect if it was directly responsible for the observed effects of diet.

Alternately, higher concentrations of PUFA in cell and organelle membranes are associated with greater enzyme activity and performance (Turner et al. 2006; Maillet and Weber 2007; Giroud et al. 2013). Higher concentrations of 18:2n6 in the muscle membranes of birds fed the 32% diet and correspondingly higher activity of oxidative and transport enzymes could thus explain the higher metabolism observed in that group early in the experiment, although once again these concentrations were stable throughout the experiment whereas performance changed. It is also unclear why changes in membrane and membrane-bound enzyme properties would affect birds differently depending on the fat load carried during flight. Finally, membrane 18:2n6 concentration was not related to any measure of performance, as would be

expected if membrane properties were directly responsible for the observed effects of diet.

Fatty acids, especially PUFAs, are also known to have a number of signaling functions, acting as ligands for Peroxisome Proliferator Activated Receptors (PPARs) and precursor molecules for eicosanoid hormones (Sampath and Ntambi 2004; Nagahuedi et al. 2009; Corder et al. 2016). PPARs, in particular, are involved in the regulation of fat oxidation (Wolfrum and Spener 2000; Weber 2011; Demoranville et al. 2019), and so upregulation of these pathways after exposure to higher dietary concentrations of 18:2n6 could result in greater oxidative capacities for birds in the 32% group, again matching the results early in this experiment. Concentration-dependent negative feedback to 18:2n6 exposure could then explain diet-specific changes in metabolism over time (Inoue et al. 2000; Fujimori 2012). It is also plausible that diet-specific changes in metabolism over time and fat loads can be explained by integrating innate circannual- and body condition-based influences on the regulation of oxidative capacity (Batista-Pinto et al. 2009; McFarlan et al. 2009; Zhang et al. 2015b; Corder et al. 2016). Finally, signaling effects are indirect and so do not require correlations between tissue PUFA composition and performance.

Of the three hypotheses discussed here, none are clearly rejected based on the results of this study. However, the fuel hypothesis does not adequately explain changes over time, the membrane hypothesis does not adequately explain changes over fat loads, and both predict correlations between tissue fatty acid composition and performance that were absent. On balance, it appears that the signaling hypothesis is best supported and future studies should seek to test the mechanistic links (e.g. PPAR

density, oxidative enzyme density and activity) between diet and whole-animal performance that this hypothesis predicts.

Dietary Antioxidants and Whole-Animal Performance

The accumulation of oxidative damage from the byproducts of aerobic metabolism is expected to reduce whole-animal performance either directly, through reduced cellular and organismal function (Eikenaar et al. 2018), or indirectly, as resources are directed to endogenous antioxidant responses rather than oxidative capacity (Cooper-Mullin and McWilliams 2016; Larcombe et al. 2017). The potential for reduced performance is particularly high for individuals with high tissue PUFA concentrations, due to elevated risk of peroxidation (Hulbert 2010; Skrip and McWilliams 2016). Dietary antioxidants have been proposed to relieve these tradeoffs (Catoni et al. 2008b; Larcombe et al. 2008; Skrip et al. 2016), by either enhancing preparation for oxidative stress or speeding recovery and preventing the accumulation of damage (Beaulieu and Schaefer 2013).

In contrast to this prediction, we found no influence of dietary antioxidants on any of our measures of whole-animal performance, possibly as a result of a low accumulation of oxidative damage from flight training and/or the high-PUFA diet. It is also possible that the hydrophilic antioxidants used to supplement the diets simply did not remain in circulation at high enough concentrations to prophylactically counter oxidative damage and failed to support recovery from oxidative damage (Beaulieu and Schaefer 2013; Cooper-Mullin and McWilliams 2016). One other explanation is that endogenous antioxidants such as uric acid (Machin et al. 2004; Alan and McWilliams 2013) could be preferentially used to counter high RS

production. An emphasis on uric acid production would help explain increasing rates of lean tissue catabolism over the course of the experiment in the 32% diet group, although circulating uric acid concentrations did not themselves differ between diet groups either before or after flight. Future measurements of antioxidant capacity and oxidative damage may better distinguish between these possibilities.

Interrelations between Basal, Peak, and Sustained Metabolism

As with a number of previous studies (McKechnie and Swanson 2010; Swanson et al. 2012; Barceló et al. 2017), we found no correlation between BMR and PMR or between either and any of our measures of sustained metabolism in the wind tunnel. This discontinuity suggests that basal, sustained, and peak metabolism are regulated differently from one another, albeit with a shared, underlying link to diet. Variation in BMR can result from variation in the relative sizes and metabolic activities of a wide range of tissues (Vézina and Williams 2005; Konarzewski and Kijazek 2013), and so the influence of diet on BMR in this study may have resulted from variation in the metabolic activity of the liver and other tissues that are always in use. In contrast, peak metabolism during physical activity is much more closely related to the capabilities of skeletal muscle and fuel transport (Wiersma et al. 2007; Zhang et al. 2015a), and for birds is likely limited by the abundance of oxidative enzymes and lipid transporters in flight muscle (Jenni and Jenni-Eiermann 1998; Vézina and Williams 2005; Barceló et al. 2017). The metabolic activity of both skeletal muscle and visceral organs can be influenced by fatty acid-based signaling pathways (Sampath and Ntambi 2004; Batista-Pinto et al. 2009; Corder et al. 2016).

Sustained metabolism during physical activity is similarly related to the characteristics of skeletal muscle and fuel supply, but is regulated below maximal levels (Hammond and Diamond 1997; Wiersma et al. 2007). This regulation is perhaps most readily modulated at either the initial release of triacylglycerols by lipases in adipose tissue or by negative feedback of transport and oxidation in the mitochondria (Hammond and Diamond 1997; McClelland 2004; Weber 2011). Both of these steps can be regulated by fatty acid-based signaling pathways (Kim and Lee 2010; Corder et al. 2016) and could thus result in diet-dependent regulation of sustained fat catabolism during physical activity. Upregulation of fat catabolism could, in turn, downregulate lean catabolism (Jenni and Jenni-Eiermann 1998; Yeo et al. 2008; Gerson and Guglielmo 2013), leading to the inverse relationship observed between the two.

Diet, Whole-Animal Performance, and Songbird Migratory Ecology

Migration is an energy-intensive life history stage and failure to meet these energetic challenges can lead to high rates of mortality during migratory periods (Wikelski et al. 2003; Dingle 2014; Rockwell et al. 2017; Ward et al. 2018). Over the course of a migration, relatively small differences in metabolism could accumulate and lead to substantial differences in the energetic requirements and success of migrants. As with previous studies (Pierce et al. 2005; McWilliams and Pierce 2006; Price and Guglielmo 2009), our results demonstrate that dietary fatty acids are capable of producing such differences in metabolism, with dietary 18:2n6 apparently mediating many of these effects. However, our finding of variable dietary effects over time

means that the benefits of a given diet are likely to be dependent on context. In the shorter term, a diet high in 18:2n6 led to less efficient energy use during flight, but also lower rates of lean catabolism which could help preserve muscle tissue during flight and lead to faster recovery post-flight. Similarly, if the elevated BMR of the 32% 18:2n6 group early in the experiment was the result of larger or more metabolically active digestive organs, it may be indicative of an increased ability to gain mass in preparation for migratory flights. In contrast, the decreased BMR, more energy efficient flight, and higher protein catabolism resulting from prolonged exposure to a high 18:2n6 diet could provide the energy savings necessary to complete migratory flights under energetic constraints. The time course of these effects could potentially be aligned with the migratory period to maximize benefits: fattening and maintaining muscle mass would be most important early in migration, while efficiency could be most important for ensuring survival later in migration (Smith and McWilliams 2014; Ward et al. 2018). It is important, however, to note that it is unclear how efficiency during wind-tunnel flight translates into behavior and efficiency in the wild when birds are less constrained and can choose their speed and flight space (Rothe et al. 1987; Jenni-Eiermann et al. 2002; Engel et al. 2006). Future studies may find it profitable to investigate the effects of dietary fatty acids without these constraints. Fatty acids in the n6 family, including 18:2n6, are found more commonly in terrestrial ecosystems than aquatic systems (Klasing 1998; Martinez del Rio and McWilliams 2016), and are found in high concentrations in many of the fruits that migratory songbirds consume during fall migration (Zurovchak 1997; Smith et al. 2007; Boyles 2011). Thus, songbirds may be able to selectively

manipulate their dietary intake of 18:2n6, and closely match their metabolism to the needs of their current life history stage.

Conclusion

In this study we directly tested the effect of dietary 18:2n6 on whole-animal performance and demonstrated that it influences the basal, peak, and sustained metabolism of European Starlings. However, in contrast to previous studies, the influence of diet changed over the course of the experiment with the 32% 18:2n6 diet associated with higher metabolism early in the experiment and lower metabolism late in the experiment. In contrast the 13% 18:2n6 diet was associated with metabolic rates that were more stable or demonstrated an increasing trend over the course of the experiment. We conclude that these patterns are best explained by signaling properties of 18:2n6 and its derivatives, which led to an upregulation of fat metabolism early in the experiment and then either experienced negative feedback with prolonged exposure or integrated innate circannual changes in metabolism in a diet-dependent manner. The diverse and indirect signaling effects of 18:2n6 also help to explain the apparent independence of its influence on basal, peak, and sustained metabolism. Contrary to our expectations, we found no influence of dietary antioxidants on whole-animal metabolism, indicating either a lack of substantial oxidative stress or an inability to effectively route hydrophilic antioxidants to the sites of damage prophylactically or palliatively. This study again highlights the importance of dietary fatty acids for songbird performance and suggests that they could strongly

influence songbird ecology especially given their availability in terrestrial food sources.

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Tables

Table 1. Ingredients and fatty acid composition of semisynthetic diets used in this study.

Ingredients	% of Dry Mass	Oil Mixture (%)	
		High 18:2n6	Low 18:2n6
Agar ^a	3.19		
Casein ^a	19.12		
Cellulose ^a	4.97		
D-glucose ^b	39.18		
Amino Acid Mix ^c	2.68		
Salt Mix ^d	4.78		
Vitamin Mix ^d	0.38		
Elderberry Powder ^e	0.42		
Mealworms ^f	6.16		
Plant oils ^g	19.12		
Fatty Acid ^h :			
12:0		0.24	0.21
14:0		0.38	0.91
16:0		8.65	29.64
16:1n7		0.3	0.41
18:0		2.83	3.9
18:1n9		45.2	42.76
18:2n6		31.87	13.86
18:3n3		4.05	2.68
20:1n9		0.55	0.4
20:4n6		0	0
22:6n3		0.15	0.11
24:1		0.1	0.08

^a U.S. Biomedical Corp., Cleveland, OH; ^b Fisher Scientific, Waltham, MA; ^c Assembled after Murphy and King (1982) from individual amino acids supplied by Fisher Scientific; ^d MP Biomedicals, Santa Ana, CA; ^e High antioxidant diets only: Artemis International, Fort Wayne, IN; ^f Freeze dried: Exotic Nutrition, Newport News, VA; ^g low 18:2n6 diet: canola oil and palm oil, high 18:n6 diet: canola oil and sunflower oil, supplied by Jedwards International, Braintree, MA; ^h Diet fatty acid concentrations (percent by mass) was measured by gas chromatography in lipids extracted from the diets. Only the twelve most concentrated fatty acids are listed.

Table 2. Parameter estimates and Wald statistics for final models of BMR and PMR.

The diet with high 18:2n6 and high antioxidants is the reference group for both models. Colons indicate interactions between model effects and significant p-values are in bold.

Coefficients:	Estimate	SE	<i>t</i> - value	<i>P</i>
<u>BMR</u>				
Intercept	162.414	69.268	2.345	0.0219
Body Mass	0.614	0.585	1.049	0.2980
Date	-0.261	0.169	-1.542	0.1277
Dietary FA	-218.381	67.131	-3.253	0.0018
Sex	4.745	4.929	0.963	0.3391
Dietary AOX	4.848	3.883	1.249	0.2160
Date : Dietary FA	0.724	0.224	3.228	0.0019
$R^2 = 0.194, df = 69$				
<u>PMR</u>				
Intercept	1945.869	829.083	2.347	0.0218
Body Mass	19.344	6.636	2.915	0.0048
Time	-930.181	636.812	-1.461	0.1487
Date	-4.190	1.957	-2.141	0.0358
Dietary FA	-1625.900	749.705	-2.169	0.0336
Sex	-59.024	55.870	-1.056	0.2945
Dietary AOX	26.253	43.240	0.607	0.5458
Date : Dietary FA	5.308	2.505	2.118	0.0378
$R^2 = 0.196, df = 68$				

Table 3. Parameter estimates and Wald statistics for final models of flight duration, fat catabolism, lean catabolism, and energy expenditure. The low 18:2n6, high antioxidant diet is the reference group for all models. Significant p-values are in bold.

Coefficients:	Estimate	SE	<i>t</i> - value	<i>P</i>
<u>Flight Duration</u>				
Intercept	1486.355	456.228	3.258	0.0027
Body Mass	1.159	1.669	0.694	0.4925
Pre-Flight Fat Mass	-358.326	101.566	-3.528	0.0013
Date	-4.714	1.441	-3.272	0.0026
Dietary FA	-2147.992	970.870	-2.212	0.0342
Sex	-15.639	16.551	-0.945	0.3518
Dietary AOX	-10.929	10.891	-1.004	0.3231
Pre-Flight Fat Mass: Date	1.242	0.325	3.820	0.0006
Pre-Flight Fat Mass: Dietary FA	521.563	256.455	2.034	0.0503
Date : Dietary FA	6.739	3.004	2.244	0.0319
Pre-Flight Fat Mass : Date : Dietary FA	-1.643	0.788	-2.084	0.0452
$R^2 = 0.747, df = 32$				
<u>Fat Catabolism</u>				
Intercept	4.220e-03	4.970e-03	0.849	0.4018
Body Mass	1.613e-04	4.326e-05	3.730	0.0007
Pre-Flight Fat Mass	8.695e-05	1.376e-04	0.632	0.5318
Date	-1.441e-05	1.125e-05	-1.280	0.2090
Dietary FA	1.751e-02	5.265e-03	3.326	0.0021
Sex	-6.013e-04	4.272e-04	-1.408	0.1684
Dietary AOX	1.127e-04	2.798e-04	0.403	0.6896
Pre-Flight Fat Mass: Dietary FA	7.506e-04	2.601e-04	2.885	0.0067
Date : Dietary FA	-6.480e-05	1.700e-05	-3.812	0.0006
$R^2 = 0.670, df = 34$				
<u>Lean Catabolism</u>				
Intercept	7.088e-03	5.423e-03	1.307	0.1998
Body Mass	-4.764e-05	4.718e-05	-1.010	0.3195
Pre-Flight Fat Mass	-2.931e-04	1.340e-04	-2.187	0.0355
Date	4.396e-07	1.225e-05	0.036	0.9716
Dietary FA	-1.290e-02	5.741e-03	-2.248	0.0310

Sex	7.151e-04	4.630e-04	1.545	0.1314
Dietary AOX	5.645e-04	3.055e-04	1.848	0.0731
Date : Dietary FA	4.118e-05	1.799e-05	2.289	0.0282
$R^2 = 0.379, df = 35$				
<u>Energy Expenditure</u>				
Intercept	0.2975	0.2121	1.403	0.1697
Body Mass	0.0054	0.0018	2.950	0.0057
Pre-Flight Fat Mass	-0.0010	0.0059	-0.163	0.8717
Date	-0.0006	0.0005	-1.194	0.2407
Dietary FA	0.4607	0.2246	2.051	0.0481
Sex	-0.0117	0.0182	-0.643	0.5243
Dietary AOX	0.0142	0.0119	1.192	0.2415
Pre-Flight Fat Mass: Dietary FA	0.0265	0.0111	2.386	0.0227
Date : Dietary FA	-0.0018	0.0007	-2.458	0.0192
$R^2 = 0.563, df = 34$				

Table 4. Fatty acid composition of pectoralis muscle lipid droplets (Neutral lipid fraction) and membranes (Polar lipid fraction) of starlings fed diets differing in 18:2n6 concentration. Composition of the ten most concentrated fatty acids is presented as mean percent of total fatty acids by mass $\pm$ standard deviation.

Lipid Fraction	Diet	Fatty Acid	Cohort 1 54 - 64 Days on Diet	Cohort 2 66 -76 Days on Diet	Cohort 3 78 - 88 Days on Diet	Cohort 4 90 - 100 Days on Diet	Cohort 5 102-112 Days on Diet
Neutral	13%	C14:0	1.09 $\pm$ 0.19	1.14 $\pm$ 0.33	1.13 $\pm$ 0.14	0.75 $\pm$ 0.08	0.96 $\pm$ 0.2
		C15:1	1.09 $\pm$ 0.19	1.09 $\pm$ 0.24	1.31 $\pm$ 0.13	0.97 $\pm$ 0.06	1.04 $\pm$ 0.21
		C16:0	19.06 $\pm$ 1.46	18.5 $\pm$ 1.38	20.53 $\pm$ 1.15	20.88 $\pm$ 0.61	24.89 $\pm$ 0.87
		C16:1	1.53 $\pm$ 0.14	1.6 $\pm$ 0.24	1.64 $\pm$ 0.1	1.7 $\pm$ 0.14	1.35 $\pm$ 0.27
		C18:0	5.11 $\pm$ 0.32	5.33 $\pm$ 0.49	4.9 $\pm$ 0.23	5.37 $\pm$ 0.08	6.13 $\pm$ 0.29
		C18:1	43.3 $\pm$ 1.58	42.95 $\pm$ 2	42.99 $\pm$ 0.86	46.51 $\pm$ 1.21	42.39 $\pm$ 0.72
		C18:2	11.23 $\pm$ 1.22	10.18 $\pm$ 0.27	9.9 $\pm$ 0.42	11.78 $\pm$ 0.54	10.33 $\pm$ 0.26
		C18:3	0.86 $\pm$ 0.09	0.82 $\pm$ 0.09	0.9 $\pm$ 0.12	1.1 $\pm$ 0.1	0.96 $\pm$ 0.21
		C20:4	0.43 $\pm$ 0.1	0.74 $\pm$ 0.23	0.53 $\pm$ 0.09	0.39 $\pm$ 0.03	0.33 $\pm$ 0.08
		C22:6	0.36 $\pm$ 0.08	0.68 $\pm$ 0.23	0.43 $\pm$ 0.08	0.31 $\pm$ 0.04	0.24 $\pm$ 0.06
	32%	C14:0	1.56 $\pm$ 0.31	1.49 $\pm$ 0.16	1.21 $\pm$ 0.2	0.88 $\pm$ 0.1	0.86 $\pm$ 0.09
		C15:1	1.79 $\pm$ 0.4	1.43 $\pm$ 0.12	1.6 $\pm$ 0.28	1.2 $\pm$ 0.15	1.03 $\pm$ 0.1
		C16:0	9.98 $\pm$ 0.5	15 $\pm$ 0.72	11.53 $\pm$ 0.66	11.19 $\pm$ 0.62	10.56 $\pm$ 0.43
		C16:1	1.16 $\pm$ 0.06	1.11 $\pm$ 0.08	1.29 $\pm$ 0.09	1.17 $\pm$ 0.11	1.16 $\pm$ 0.05
		C18:0	4.76 $\pm$ 0.25	4.62 $\pm$ 0.21	4.99 $\pm$ 0.22	4.94 $\pm$ 0.23	4.54 $\pm$ 0.22
		C18:1	40.87 $\pm$ 1.39	39.17 $\pm$ 1.57	42.48 $\pm$ 1.11	42.78 $\pm$ 0.46	43.38 $\pm$ 0.93
		C18:2	18.64 $\pm$ 1.02	11.97 $\pm$ 0.54	20.35 $\pm$ 0.94	21.55 $\pm$ 0.42	21.08 $\pm$ 0.44
		C18:3	1.03 $\pm$ 0.15	1.08 $\pm$ 0.08	1.34 $\pm$ 0.18	1.54 $\pm$ 0.11	1.37 $\pm$ 0.08
		C20:4	0.71 $\pm$ 0.16	0.66 $\pm$ 0.08	0.75 $\pm$ 0.15	0.64 $\pm$ 0.08	0.57 $\pm$ 0.06
		C22:6	0.58 $\pm$ 0.15	0.59 $\pm$ 0.08	0.53 $\pm$ 0.17	0.42 $\pm$ 0.06	0.43 $\pm$ 0.08
Polar	13%	C14:0	0.08 $\pm$ 0.02	0.15 $\pm$ 0.03	0.14 $\pm$ 0.03	0.07 $\pm$ 0.02	0.09 $\pm$ 0.02
		C15:1	0.07 $\pm$ 0.01	0.1 $\pm$ 0.02	0.11 $\pm$ 0.03	0.06 $\pm$ 0.01	0.07 $\pm$ 0.01
		C16:0	21.25 $\pm$ 0.83	19.78 $\pm$ 0.47	20.12 $\pm$ 0.7	21.26 $\pm$ 0.81	21.86 $\pm$ 1.16

		C16:1	0.19±0.02	0.24±0.02	0.2±0.01	0.14±0.03	0.16±0.03
		C18:0	22.13±0.3	22.19±0.48	22.19±0.53	22.29±0.49	22.25±0.17
		C18:1	10.72±0.36	10.16±0.57	10.79±0.27	10.49±0.3	9.78±0.2
		C18:2	12.27±0.55	10.72±0.45	13.24±0.39	12.45±0.34	13.44±0.3
		C18:3	0.43±0.05	0.45±0.07	0.59±0.04	0.51±0.07	0.62±0.03
		C20:4	9.56±0.33	9.57±0.55	10.17±0.33	9.02±0.41	10.05±0.22
		C22:6	11.09±0.71	11.2±0.84	9.69±0.34	10.71±0.87	10.88±0.62
	32%	C14:0	0.17±0.04	0.09±0.01	0.13±0.03	0.74±0.69	0.14±0.05
		C15:1	0.12±0.02	0.07±0	0.11±0.02	0.23±0.15	0.13±0.05
		C16:0	16.41±0.78	17.57±0.56	18.77±0.53	15.96±0.89	18.18±0.95
		C16:1	0.17±0.01	0.16±0.01	0.17±0	0.26±0.12	0.18±0.03
		C18:0	24.82±0.64	23.54±0.39	23.95±0.39	22.62±1.52	22.89±0.79
		C18:1	8.55±0.31	9.03±0.31	8.13±0.16	7.65±0.61	9.22±0.47
		C18:2	16.28±0.69	14.5±0.67	15.94±0.64	15.61±1.09	16.95±0.56
		C18:3	0.53±0.05	0.6±0.04	0.59±0.04	0.52±0.04	0.6±0.04
		C20:4	9.38±0.48	9.81±0.28	10.05±0.39	8.09±0.77	8.88±0.24
		C22:6	10.73±0.77	11.85±0.52	9.65±0.34	8.81±0.69	10.81±0.9

Figures

Figure 1. Basal (BMR) and peak (PMR) metabolic rates of starlings fed either high (32% of total FA, $N = 40$) or low (13%, $N = 36$) amounts of 18:2n6. Metabolic scope refers to the quotient of PMR/BMR and shaded areas indicate 95% confidence intervals of regressions. Julian date 269 to 324 is 27 Sept to 20 Nov 2015 and so is 26 to 81 days after being introduced to the four experimental diets on 1 Sept.

Figure 2. Flight durations of starlings fed low (13%, A) or high (32%, B) concentrations of 18:2n6 during a voluntary flight of >120 minutes at the culmination of the 15-day flight training regimen. Pre-flight fat mass (+/- SE) was measured using a QMR.

Figure 3. Rates of fat and lean catabolism during flight for Starlings fed low (13%, A and C) or high (32%, B and D) concentrations of 18:2n6. Pre-flight fat mass was measured using a QMR. Rates of fat and lean catabolism were calculated by dividing the fat or lean mass lost during a long flight of >120 minutes by the duration of the flight.

Figure 4. Rates of energy expenditure during flight for starlings fed low (13%, A) or high (32%, B) concentrations of 18:2n6. Pre-flight fat mass was measured with a QMR. Energy expenditure (kJ/min) was calculated by multiplying the fat and lean mass lost over the course of a >120-minute flight by their respective energy densities, summing them, and dividing by the duration of the flight.

Figure 5. Fatty acid composition of experimental diets and pectoralis muscle lipid droplets (Neutral, A) and membranes (Polar, B) in starlings. Composition is presented for the ten most concentrated fatty acid as the percent of total fatty acids by mass. Asterisks denote significant differences between diet groups ($P < 0.05$).

Figure 6. The effects of dietary linoleic acid (18:2n6) and wind-tunnel flight training on the composition of palmitic acid (16:0, A) and 18:2n6 (B) in the lipid droplets of flight muscle in European Starlings. Palmitic acid also showed significant diet-specific changes in concentration over time (C). Composition is presented as the percent of total fatty acids by mass.

Figure 7. The effects of dietary linoleic acid (18:2n6) and wind-tunnel flight training on the composition of palmitic acid (16:0, A), docosahexaenoic acid (22:6n3, B), stearic acid (18:0, C), and 18:2n6 (D) in the membranes of flight muscle in European Starlings. Stearic acid (E) and linoleic acid (F) also showed significant diet-independent changes in concentration over time. Composition is presented as the percent of total fatty acids by mass.

Figure 8. Log-transformed concentrations of β -hydroxybutyrate (BUTY), triglycerides (TRIG), and uric acid (UA) in the plasma of starlings before and after the culminating flight of a 15-day flight training regimen. There were significant increases in BUTY and UA and decreases in TRIG between pre-and post-flight indicating significant catabolism of fat and protein during the longest-duration flights.

MANUSCRIPT II:

Dietary linoleic acid, antioxidants, and flight training influence the activity of
oxidative enzymes in European Starlings (*Sturnus vulgaris*)

by

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Abstract

Multiple studies have demonstrated that diet (e.g., fatty acid composition, antioxidants) and exercise training affect the metabolic performance of songbirds during aerobic activity, although the physiological mechanisms that cause such an effect remain unclear. We tested the hypothesis that dietary linoleic acid (LA, 18:2n6) and anthocyanins (a hydrophilic antioxidant class) influence the activity and expression of oxidative enzymes in flight and leg muscle of European Starlings (*Sturnus vulgaris* N=96), a subset of which were flown over 15 days in a wind tunnel. Carnitine palmitoyl transferase (CPT) and citrate synthase (CS) activity displayed LA-dependent relationships with soluble protein concentration. Lactate dehydrogenase (LDH) was similarly related to protein concentration, although also dependent on dietary anthocyanins and flight training. 3-Hydroxyacyl CoA Dehydrogenase (HOAD) activity and protein concentration were related to date in flight muscle, but were more related to dietary anthocyanins in the leg muscle. Training also produced additive increases in CPT and leg muscle HOAD activity. FAT/CD36 expression was related to both dietary LA and training and changed over the course of the experiment. These results demonstrate a notable influence of dietary LA, anthocyanins, and training on the activity of these key oxidative enzymes, and particularly CPT and CS. Such influence suggests a plausible mechanism linking diet quality and metabolic performance in songbirds.

Keywords

linoleic acid, oxidative enzyme, citrate synthase, CPT, HOAD, LDH, FAT/CD36

Introduction

Diet quantity and quality have been long recognized to exert substantial influence on the performance of individual animals, ranging from effects on growth to immunocompetence, capacity for physical exertion, and social behavior (Klasing 1998; McDonald et al. 2011; Lihoreau et al. 2015). Diet can influence whole-animal performance in several ways: it can change the supply of raw materials used to fuel metabolism and the growth of new tissue, it can change the structural and functional properties of existing tissue as old molecules turn over, or it can start signaling cascades to change the regulation of tissue structure and function. Studies that manipulated dietary fatty acid composition have found subsequent effects on organism-level growth (Twining et al. 2016b), metabolic rates (Pierce et al. 2005; Price and Guglielmo 2009), endurance (Ayre and Hulbert 1997), energetic efficiency (McWilliams and Pierce 2006), and maximum speed (McKenzie and Higgs 1998). Such effects have been observed in a wide range of species, but have been particularly notable in songbirds (Martinez del Rio and McWilliams 2016; Twining et al. 2016a). Despite these proof-of-concept demonstrations, manipulations of dietary fatty acids have produced inconsistent effects on energy metabolism when studies used different species, used birds of different ages, and manipulated different fatty acids (e.g. BMR: Pierce et al. 2005; Amitai et al. 2009; Price and Guglielmo 2009; Twining et al. 2016b). This inconsistency highlights the need for a more thorough understanding of the mechanisms that may link dietary fatty acid composition with organism-level performance.

Changes in the supply and catabolism of fatty acids for energy by one of several mechanisms are hypothesized to link dietary fatty acid composition to energy metabolism in songbirds (Guglielmo 2010; Price 2010; Pierce and McWilliams 2014). While such changes could result from the properties of the fatty acids themselves (Raclot 2003; Price et al. 2008; Mustonen et al. 2009), they could also be the result of differing activities of rate-limiting transport proteins and catabolic enzymes responsible for shepherding fatty acids to the mitochondria and breaking them down to produce ATP (McClelland 2004; Weber 2011). Specifically, the activity of these transporters and enzymes could be indirectly increased by upregulating their expression with the activation of peroxisome proliferator-activated receptor (PPAR) pathways by polyunsaturated fatty acids (PUFA; Sampath and Ntambi 2004; Marion-Letellier et al. 2016). Alternately, the activity of transporters and enzymes that are integrated into cell and mitochondrial membranes could be directly increased by changes in the properties of those membranes following the incorporation of greater proportions of PUFA (Turner et al. 2006; Maillet and Weber 2007; Giroud et al. 2013). Previous studies have tested the effect of certain dietary combinations of fatty acids and certain long-chain PUFA on the activity of these oxidative enzymes (Nagahuedi et al. 2009; Price and Guglielmo 2009; Dick and Guglielmo 2019), but they have not tested the specific effect of linoleic acid (18:2n6), a PUFA acid implicated by several studies of organism-level performance (Ayre and Hulbert 1997; McKenzie and Higgs 1998; Pierce et al. 2005).

Diets and tissues containing high concentrations of PUFA relative to saturated (SFA) and monounsaturated fatty acids (MUFA) are more susceptible to oxidative

damage than those with lower relative concentrations (Hulbert 2010; Skrip and McWilliams 2016). This risk, combined with the proximity of lipid transporters and oxidative enzymes to reactive species production in the mitochondria creates a potential tradeoff in which PUFA-derived enhancements to energy metabolism are limited by increased damage to those transporters and enzymes. However, this tradeoff could be mitigated by the supplementation of dietary antioxidants, which could either prophylactically prevent damage or enhance recovery following damage (Larcombe et al. 2008; Beaulieu and Schaefer 2013; Cooper-Mullin and McWilliams 2016). Additionally, many of the high-PUFA foods eaten by terrestrial songbirds during migration also contain high concentrations of antioxidants (Bolser et al. 2013; Alan and McWilliams 2013). Thus, it is important to consider potential interactions with dietary antioxidants when testing the effects of dietary PUFA on the activity of fatty acid transporters and oxidative enzymes.

The properties and capabilities of muscle are also dependent on the extent to which they are used. Differences in use are therefore reflected in variation in lipid transport and catabolism among muscles (Siu et al. 2003; Zhang et al. 2015b) and increases in lipid transport and catabolism following training (Helge et al. 2001; Anttila et al. 2010; Zhang et al. 2015a). For many songbirds, such training may provide critical preparation for migratory flights (Lundgren and Kiessling 1986; Guglielmo et al. 2002), and complement fatty acid-derived changes to their capacity for energy metabolism. The elevated metabolism required by training also increases the production of pro-oxidant reactive species (Mataix et al. 1998; Larcombe et al. 2008; Jenni-Eiermann et al. 2014), potentially increasing the need for dietary

antioxidants to counter damage to tissues. Therefore, we tested the interacting effects of dietary 18:2n6, hydrophilic dietary antioxidants, and training on the activity and expression of a range of lipid transporters and oxidative enzymes in the flight and leg muscle of a songbird. Specifically, we considered the following hypotheses: 1) elevated dietary 18:2n6 increases activity and expression of transporters and oxidative enzymes, 2) elevated dietary antioxidants reduce tissue damage and increase enzyme activity and expression, 3) flight training increases enzyme activity and expression, 4) dietary antioxidants mitigate the oxidative damage tradeoffs with PUFA and training and thus produce higher enzyme activity and expression when supplementary antioxidants are combined with elevated dietary 18:2n6 and/or training. In testing these hypotheses, we selected key lipid transporters and oxidative enzymes involved in a wide range of functions during lipid metabolism, including transport into the muscle cell (fatty acid translocase, FAT/CD36), transport into the mitochondrion (carnitine palmitoyl transferase, CPT), β -oxidation (3-hydroxyacyl dehydrogenase, HOAD), and the citric acid cycle (citrate synthase, CS), as well as lactate dehydrogenase (LDH) as an indicator of anaerobic metabolic capacity.

Methods

Experimental Design

This experiment was conducted between August and December 2015 at the Advanced Facility for Avian Research (AFAR) at the University of Western Ontario (UWO) as part of a larger study on the exercise physiology of songbirds. Animal care protocols were approved by both UWO (2010-216) and the University of Rhode Island (URI;

AN11-12-009). The animals used in this study were 108 hatch-year European Starlings (*Sturnus vulgaris*), captured between August 19-23 at a dairy farm approximately 20 km north of UWO in Middlesex Center, ON and housed in four large indoor aviaries at AFAR (two 2.4m x 3.7m x 3.1m and two 2.4m x 2.3m x 3.5m). Once all birds were captured, we collected morphological measurements for each individual and randomly resorted them into four groups with roughly equal distributions of body mass and molt score (0 – 75; Ginn and Melville 1983). From capture onward, birds had *ad libitum* access to one of two semi-synthetic diets that differed only in fatty acid (FA) composition and produced reliable differences in tissue FA composition (table 1; Carter et al. *in review*): birds in two aviaries received a 32% 18:2n6 diet and two others received a 13% 18:2n6 diet. On September 1, we began adding supplementary anthocyanins (elderberry powder; Artemis International, Fort Wayne, IN) to the diets of one 32% 18:2n6 and one 13% 18:2n6 aviary, producing a 2 X 2 factorial diet manipulation with four groups: 32% 18:2n6 high anthocyanin ($N = 28$), 32% 18:2n6 low anthocyanin ($N = 27$), 13% 18:2n6 high anthocyanin ($N = 27$), and 13% 18:2n6 anthocyanin AOX ($N = 26$). We maintained aviaries at 21°C on a natural light cycle at London, Ontario and each week we weighed and inspected all birds to assess health.

On September 21, we fixed the light cycle at 11h light : 13h dark and assigned the birds to twenty single-diet cohorts of 5-6 individuals based on molt score. Beginning on September 23, and continuing every three days thereafter in descending order of molt score, we removed birds of the appropriate cohort from their aviary and placed them in individual 0.6m x 0.5m x 0.5m cages to begin a 26-day process of

flight training. Details about flight training can be found in our previous publication (Carter et al. *in review*). Briefly, birds remained in individual cages for days 1-4 following cohort separation while we measured their food intake and metabolic rates. On day five, we returned two randomly selected control (untrained) birds to their original aviary and moved the remaining (trained) birds to a 0.8m x 1.5m x 2m flight aviary. Trained birds were acquainted with the AFAR wind tunnel for 20 minutes each day on days 6-9 and then spent days 10-24 in a fifteen-day training regimen that consisted of increasing periods of flight (20min – 180min) in the wind tunnel and culminated in a flight on day 24 that lasted as long as birds would voluntarily fly, up to 6 hrs. The mean length of this final flight was 193 minutes and the maximum lasted 360 minutes. At the same time of day (1400hr – 1500hr) on days 25 and 26, respectively, untrained and trained birds were euthanized by cervical dislocation while under isofluorane anesthesia and were dissected for tissue samples that were flash frozen in liquid nitrogen and stored at -80°C until analysis. Tissue samples included pectoral and leg muscle, heart, proventriculus, gizzard, small intestines, liver, kidney, pancreas, heart, and adipose tissue, although only the first two were used for the analyses described below. This sampling schedule allowed us to compare enzyme activities in selected tissues of untrained (control) birds and trained birds which had ca. 48 hrs to recover from their longest flight on day 24.

Enzyme Assays

The activities of the enzymes CPT (EC 2.3.1.21), CS (EC 2.3.3.1), HOAD (EC 1.1.1.35), and LDH (EC 1.1.1.27) in pectoral ($N = 95$) and leg ($N = 91$) muscle were

assayed following the methods of (Price et al. 2010; Zajac et al. 2011), modified to be analyzed with a plate reader (Synergy HTX, Biotek Instruments, Winooski VT USA). All reagents used in these assays were obtained from Fisher Scientific (Waltham MA USA). Briefly, we homogenized approximately 100mg of muscle tissue in 9 volumes of homogenization buffer (20mM Na_2HPO_4 , 0.5 mM EDTA (, 0.2% BSA, 50% glycerol, 0.1% triton x-100, 50 $\mu\text{g}/\text{ml}$ Aprotinin, pH 7.4) with 3 x 10 sec pulses of a high-speed stainless-steel homogenizer (Tissue Master 125, Omni International, Kennesaw GA USA). Samples were kept on ice for at least 30 sec in between pulses and crude homogenate was diluted 1:100 in homogenization buffer before being stored at -80°C until the assays were conducted. All assays were 10-minute kinetic absorbance assays run in duplicate at 39°C with a total volume of 200 μl in 96-well plates and with the optical path length corrected to 1 cm. CPT was assayed in 50mM Tris buffer (pH 8.0) with 7.5mM carnitine, 0.15mM DTNB (5,5'-dithio-bis-[2-nitrobenzoic acid]), 0.035mM palmitoyl CoA, and 20 μl of diluted homogenate. CS was also assayed in 50mM Tris buffer (pH 8.0) with 0.75mM oxaloacetic acid, 0.15mM DTNB, 0.15mM acetyl CoA, and 2 μL of diluted homogenate. HOAD was assayed in 50mM imidazole buffer (pH 7.4) with 0.2mM NADH, 1mM EDTA, 2mM acetoacetyl CoA, and 20 μL of diluted homogenate. LDH was also assayed in 50mM imidazole (pH 7.4) with 5mM DTT (DL-dithiothretiol), 0.3mM NADH, and 2 μL of diluted homogenate. Absorbances were read at 412nm for CPT and CS and 340nm for HOAD and LDH and enzyme catalytic activity per gram of wet tissue was calculated as follows:

$$\frac{\mu mol}{min \cdot g} = \frac{\beta \times V_{well} \times D}{\varepsilon \times V_{sample}}$$

where β is the rate of change in absorbance (Δ Absorbance / min), V_{well} is the final volume of the well, D is the dilution factor of the for the homogenate, ε is the extinction coefficient (13.6 for DTNB, 6.22 for NADH), and V_{sample} is the volume of the diluted homogenate added to the well.

Soluble Protein Concentration and CS and FAT/CD36 Expression

We assayed protein concentration in diluted pectoral and leg muscle homogenate using a Bradford assay (Bio-Rad, Hercules CA USA) with a BSA standard. Although this assay is often used to quantify total protein, it does not include the insoluble protein in connective tissue making it more representative of soluble protein in our whole-tissue homogenates (López et al. 1993). We present soluble protein concentration as mg per g of wet tissue. We then used a luminescent immunoblotting procedure (Gallagher 2008; Fukasawa et al. 2010; Mowry et al. 2017), modified to be read on a plate reader, to quantify the relative concentrations of CS and FAT/CD36 proteins in a subset of pectoralis homogenate samples ($N = 24$), diluted to a soluble protein concentration of 0.5mg/ml with Tris-glycine buffer (pH 6.8). Briefly, we incubated 75 μ L of tissue homogenate overnight at 4°C in high-binding ELISA 96-well plates (Greiner Bio-One, Kremsmünster Austria) and then blocked wells for one hour at room temperature with 150 μ L of 5% BSA in PBS buffer (pH 7.4). Next, we incubated wells for three hours at room temperature with 75 μ L of a primary antibody (mouse anti-CS, NM_004077.2, or mouse anti-FAT/CD36, AB_2538312) at a

concentration of 5 μ g/mL in blocking buffer, washed 3x with 0.1% PBS triton x-100 (PBST), washed 3x with PBS, and then incubated wells overnight at 4°C with 75 μ L of secondary antibody (rabbit anti-mouse conjugated to HRP, AB_2534831) at a concentration of 5 μ g/mL in blocking buffer. Finally, the wells were again washed 3x with 0.1% PBST and 3x with PBS, then treated with 50mL of chemiluminescent reagent (Pierce ECL Western Blotting Substrate, Fisher Scientific, Waltham Ma USA) for 1 minute, and luminescence was read 2 minutes later with a plate reader (Synergy HTX, Biotek Instruments, Winooski VT USA). Four replicates were run for each sample and CS and FAT/CD36 were analyzed separately. We compared luminescence values to a standard curve of known concentrations of the primary antibody and present CS and FAT/CD36 concentrations as μ g/mg of soluble protein.

Statistical Analyses

We tested the influence of diet and training on enzyme activity, soluble protein concentration and the expression of CS and FAT/CD36 with linear models that also controlled for the effects of body size (body mass and wing chord), sex, Julian date, and soluble protein concentration (when not treated as the response). Starting with a full model of main effects and interactions between diet and training, we tested and removed non-significant interaction terms (including 3- and 2-way interactions involving date and soluble protein concentration). We did not retain main effects of body size or sex when those terms were non-significant. We tested for correlations between enzyme activity, soluble protein concentration, and the expression of CS and FAT/CD36 separately for pectoral and leg muscle and used a Bonferroni correction to

compensate for multiple tests. To better understand variation in soluble protein concentration, we tested the correlation between soluble protein and total lipid content of flight muscle (see Carter et al. *in review* for methods). All analyses were conducted in R (v3.3.2; R Core Team, Vienna, Austria).

Results

Enzyme Activity and Soluble Protein Concentration

The activities of different enzymes were related to varying combinations of dietary 18:2n6, dietary anthocyanins, training, soluble protein concentration, and date, and were also dependent on tissue type. In pectoral muscle both CPT and CS activity were positively related to soluble protein concentration when birds were fed the 13% 18:2n6 diet, but negatively related when fed the 32% 18:2n6 diet (table 2; CPT: figure 1A, $T_{87} = -2.286$, $P = 0.0247$; CS: figure 1C, $T_{86} = -3.132$, $P = 0.0024$).

Conversely, in leg muscle CPT and CS activity were positively related to soluble protein concentration when birds were fed the 32% 18:2n6 diet, but unrelated when fed the 13% 18:2n6 diet (table 3; CPT: figure 1B, $T_{83} = 2.027$, $P = 0.0458$; CS: figure 1D, $T_{83} = 1.944$, $P = 0.0553$) and negatively related to wing chord regardless of diet (table 3; CPT: $T_{83} = -2.254$, $P = 0.0268$; CS: $T_{83} = -2.633$, $P = 0.0101$). Additionally, pectoral muscle CS activity was positively related to body mass (table 2; $T_{86} = 2.201$, $P = 0.0304$) and flight training produced an additive increase in the activity of CPT in both pectoral (table 2, figure 2A; $T_{87} = -3.149$, $P = 0.0023$) and leg muscle (table 3, figure 2B; $T_{83} = -2.092$, $P = 0.0395$). LDH activity was also related to soluble protein concentration in both pectoral and leg muscle (figures 1E and 1F), but these

relationships depended on, respectively, the interaction between dietary 18:2n6 and dietary anthocyanins (table 2; $T_{84} = 2.391$, $P = 0.0190$) and the interaction between dietary 18:2n6 and flight training (table 3; $T_{81} = 3.695$, $P = 0.0004$). HOAD activity in pectoral muscle increased over the course of the experiment (table 2; figure 3A; $T_{88} = 2.457$, $P = 0.0160$), but in leg muscle only increased over time in the low anthocyanin group whereas the high anthocyanin group decreased with date (table 3; figure 3B; $T_{84} = 3.073$, $P = 0.0029$). Leg muscle HOAD activity additively increased with flight training (table 3; figure 2C; $T_{84} = -2.075$, $P = 0.0411$). Soluble protein concentration in pectoral muscle was also positively related to date (table 2, figure 3C; $T_{89} = 3.300$, $P = 0.0014$), but only exhibited additive decreases with training (table 3; $T_{86} = 2.516$, $P = 0.0137$) and dietary anthocyanins (table 3; $T_{86} = 2.163$, $P = 0.0333$) in leg muscle (figure 3D). Variation in soluble protein concentration needs to be traded off with variation in other tissue components. However, soluble protein concentration was unrelated to total lipid concentration in the flight muscle ($r_{94} = 0.045$, $P = 0.669$), suggesting that lipid content does not drive variation in soluble protein.

CS and FAT/CD36 Expression

The expression of CS in pectoral muscle was negatively related to body mass (table 4, figure 4A; $T_{16} = -2.561$, $P = 0.0209$). In contrast, FAT/CD36 expression decreased over the course of the experiment in all experimental groups except untrained birds who were fed the 13% 18:2n6 diet (table 4, figure 4B, $T_{12} = -2.327$, $P = 0.0383$).

Correlations among Enzyme Activities and Expression

In pectoral muscle (table 5) CPT activity was positively correlated with CS activity and HOAD activity. In contrast, CS expression showed substantial negative relationships with CS activity, CPT activity, and FAT/CD36 expression, although none of these relationships were significant. In leg muscle (table 6) the activity of each enzyme was positively correlated to the activity of other enzymes with the exception of the non-significant relationship between HOAD activity and LDH activity.

Discussion

We used an experimental approach to test the hypothesized effects of dietary 18:2n6, hydrophilic dietary antioxidants, and training on the activity and expression of several key lipid transporters and oxidative enzymes in a songbird. Contrary to expectations, we found that influence of diet was dependent on either the soluble protein concentration of the muscle tissue or the timing of the sample within the course of the experiment, whereas the influence of training was largely additive. We also found no evidence that elevated dietary PUFA or training produced tradeoffs for muscle metabolism that could be relieved by the supplementation of hydrophilic dietary antioxidants. We did, however, find striking differences in the activity of oxidative enzymes between flight and leg muscle, which further emphasizes the context-dependence of dietary influence on muscle metabolism and suggests that the signaling functions of PUFA are largely responsible for the effects of dietary 18:2n6.

The mechanisms implicated by these results help to shed light on previous studies that found links between dietary 18:2n6 and organism-level performance.

Dietary 18:2n6 and enzyme activity and expression

Dietary PUFA are hypothesized to influence the activity of enzymes involved in fatty acid catabolism by either upregulating their expression via PPAR pathways or by changing the properties of membranes to which some enzymes are bound (Price 2010; Pierce and McWilliams 2014). While so-called “natural doping” involving long-chain n-3 fatty acids has received mixed support (Nagahuedi et al. 2009; Guglielmo 2018; Dick and Guglielmo 2019), the influence of n-6 fatty acids, and particularly 18:2n6, has not been directly tested. Nevertheless, in the studies on songbirds where 18:2n6 concentration did differ between experimental diets no differences were found in the activity of oxidative enzymes (Price and Guglielmo 2009; Dick and Guglielmo 2019). In contrast, we found significant differences between our 32% 18:2n6 and 13% 18:2n6 groups in CPT activity, CS activity, LDH activity, and FAT/CD36 expression. Some of this qualitative difference may be related to the greater difference in 18:2n6 concentration and its exchange with a saturated fat (instead of n-3 PUFA) in our experiment, but a more likely explanation is that our study was the only one to consider the interaction between diet and soluble protein content of the muscle tissue. Rather than simple additive differences, diet groups exhibited similar rates of CPT, CS, and LDH activity at low-to-average concentrations of protein and diverged at high protein concentrations. These results suggest that 1) dietary 18:2n6 is involved in regulating the expression of CPT, CS,

and LDH, and 2) that this regulation is not independent, but occurs within the broader context of protein synthesis and degradation. Thus, although the expression of these enzymes may be upregulated via activation of PPARs (Sampath and Ntambi 2004; Collin et al. 2009), the strength of the response may be contingent upon a general increase in muscle condition, such as those associated with migration (Evans et al. 1992; Price et al. 2011).

Dietary 18:2n6 influenced CPT and CS activity similarly, with flight muscle in the 13% 18:2n6 group showing a positive relationship between enzyme activity and soluble protein and birds in the 32% 18:2n6 group showing a negative or null relationship between enzyme activity and soluble protein. This pattern suggests that, at high protein concentrations, birds fed the 13% 18:2n6 diet were more oriented towards fat catabolism and capable of higher metabolic rates than birds fed the 32% 18:2n6 diet. Such differences in metabolic orientation run counter to the expected influence of 18:2n6, but could perhaps be explained by changes in fiber type and myosin heavy chain expression (Lundgren and Kiessling 1988; Goslow, Jr. et al. 2003), which are related to the soluble protein content of muscle (Carroll et al. 2004; Goodman et al. 2012) and may be influenced by dietary PUFA (Hashimoto et al. 2016). It is also possible that mitochondrial membranes were disrupted during tissue homogenization (Dick and Guglielmo 2019), and the lower activity of CPT and CS with the 32% 18:2n6 diet in our experiment would be compensated by membrane effects *in vivo*. The correlation between CPT and CS activity suggests that the regulation of these enzymes is coordinated, perhaps reflecting their control of fatty acid transport into mitochondria and entrance into the citric acid cycle. In contrast,

the specific patterns of LDH were more complicated, involving interactions with dietary anthocyanins and training in the flight and leg muscle, respectively. This suggests that the regulatory influence of dietary 18:2n6 on glycolytic capacity is more context dependent than its influence on aerobic metabolism, only occurring when antioxidants are supplemented in the flight muscle and being reversed by training in the leg muscle. Finally, FAT/CD36 expression was elevated early in the experiment in the 32% 18:2n6 group regardless of whether the birds were trained, suggesting that 18:2n6 is straightforwardly involved in the upregulation of this transporter, in contrast with the more complicated patterns observed in CPT, CS, and LDH.

Dietary Anthocyanins and Enzyme Activity and Expression

Dietary antioxidants, including anthocyanins, have been suggested to be important regulators of oxidative damage in animals and capable of relieving oxidative tradeoffs associated with aerobic metabolism and tissue PUFA content (Catoni et al. 2008; Larcombe et al. 2008; Skrip and McWilliams 2016). However, in this study anthocyanin supplementation did not produce equivalent activity between 13% and 32% 18:2n6 or trained and untrained groups for any enzyme measured, thus providing little evidence for any such tradeoffs. Similarly, we did not see the expected increases in activity corresponding to reduced damage and inactivation with antioxidant supplementation (Kim et al. 2004; Chepelev et al. 2009). The absence of such patterns suggests that either 1) the supplemented anthocyanins did not adequately reach the mitochondria to produce substantial effects on enzyme activity, 2) reactive species (RS) production was relatively low in the sampled tissues, or 3)

RS production was already effectively countered by the birds' endogenous antioxidant system.

Nevertheless, the supplementation of diets with anthocyanins did have some influence on the activity of LDH and HOAD. With supplementary anthocyanins, the specific pattern of LDH activity resembled that seen in CPT and CS, which may suggest that regulation of anaerobic metabolism in flight muscle is similar to the regulation of aerobic metabolism, but dependent on antioxidant status. The reintroduction of oxygen following anaerobic conditions can result in elevated RS production (Halliwell and Gutteridge 2007), so the anaerobic capacity of flight muscle might be regulated differently when there is a higher supply of antioxidants to cope with this additional stress. Meanwhile, HOAD activity decreased over the course of the experiment in the leg muscle when birds were supplemented with anthocyanins, which may be the result of antioxidant-driven downregulation of mitochondrial metabolism, which has been observed in rats (Strobel et al. 2011). However, both of these explanations are highly speculative and will require further testing.

Flight Training, Muscle Tissues, and Enzyme Activity and Expression

Tissue usage has been consistently shown to influence the activity of metabolic enzymes resulting in differences between tissues (Siu et al. 2003; Zhang et al. 2015b) and between individuals who train at different rates (Helge et al. 2001; Anttila et al. 2010). Accordingly, we found positive effects of training on the activity of CPT in both tissues, HOAD activity in the leg muscle, and FAT/CD36 expression in the

flight muscle early in the experiment. These effects are consistent with training-induced increases in capacity for lipid metabolism and suggest that those increases are the result of changes in fatty acid supply rather than catabolism. Moreover, the presence of additive effects in CPT and HOAD might suggest that training coordinates the activity of these enzymes and is responsible for the correlation between them. Training also influenced the activity of LDH in the leg muscle, reversing the interaction between soluble protein content and dietary 18:2n6 from that observed in CPT and CS. Given the reorientation towards aerobic metabolism associated with endurance training (Helge et al. 1998; Siu et al. 2003; Kim et al. 2004), it is unsurprising that anaerobic capacity responded in an opposing manner, although it is unclear why this reversal did not occur in flight muscle.

As predicted, we also found substantial differences in the absolute activities of CPT, CS, and HOAD between flight and leg muscle. These findings are consistent with the central role of these enzymes in aerobic metabolism, and the known differences in aerobic work load between flight muscle and leg muscle. However, we found that flight and leg muscle also differed substantially in their responses to dietary 18:2n6, dietary anthocyanins, and flight training. In particular, leg muscle was more likely to be influenced by dietary anthocyanins (two out of three cases) and training (four out of six cases) than flight muscle. These differences might suggest that the regulation of enzyme activity in flight muscle to higher overall levels may outweigh most effects of dietary anthocyanins and training, whereas the lower regulation in leg muscle leaves such effects more visible. Flight and leg muscle also differed in how the combination of soluble protein and dietary 18:2n6 influenced the

activity of CPT and CS. Most notably, the high 18:2n6 group exhibited a negative relationship with soluble protein in flight muscle, but a positive relationship in leg muscle. Although there is no clear explanation for this reversal, one possibility is that dietary 18:2n6 consistently regulates enzyme activity to a similar level regardless of tissue when there is high soluble protein content, and so the differing trends are the result of the broad differences between tissues at low soluble protein concentrations. Future studies may find it useful to investigate the regulation of soluble protein content in different tissues and in animals fed different diets more closely.

Changes in Enzyme Activity and Expression over Time

Unexpectedly, we found several changes over the course of the experiment, including increases in flight muscle soluble protein and HOAD activity, decreases in flight muscle FAT/CD36 expression, and anthocyanin-dependent increases and decreases in leg muscle HOAD activity. It is unclear what drove these changes, but the most likely factors are time in captivity or endogenous seasonal rhythms. Multiple aspects of captivity could influence metabolic capabilities including changes in the amount or type of activity, length of exposure to the experimental diets, or adjustments to a more stable environment. However, it is not clear that such adjustments would persist throughout the experiment beyond the initial acclimation period. Additionally, it appears as though there was an inflection point in flight muscle soluble protein around November 20th that is difficult to explain with amount of time in captivity. Because photoperiod was constant throughout this experiment and birds were less than one year old, any seasonal changes would need to be entirely endogenous in

origin. Although there is some evidence for such endogenous annual rhythms (Cadee et al. 1996; Rani and Kumar 2013), they have not been explicitly linked to enzyme activity. Thus, this possibility is highly speculative.

Implications of Tissue-Level Metabolism for Organism-Level Performance

This experiment demonstrated that differences in dietary 18:2n6 content produce context-dependent differences in the activity of enzymes critical to fat-based energy metabolism. In particular, with the divergence of CPT and CS activity between the two diets at high protein concentrations, dietary fatty acid composition may be able to produce phenotypes that are more or less focused on fat catabolism. When combined with the changes in soluble protein over the course of the experiment, these phenotypic adjustments appear to be flexible over time as well as diet. Such changes over time in capacity for fat metabolism may help explain the variability of whole-animal rates of fat and lean catabolism observed in this study (Carter et al. *in review*), and also suggest that diet could be used to modify enzyme activity, and therefore tissue function, in preparation or response to challenging life history stages, such as migration. In contrast, although training and dietary anthocyanins did influence enzyme activity and expression, their effects were largely confined to the leg muscle rather than the flight muscle, and so may not be critical to preparation by songbirds for migration. To the extent that training can be used to prepare for migration, it appears to be involved in the supply of fatty acids to muscle tissue rather than changing the rate of fatty acid catabolism. Finally, all of the effects observed in this study were relatively small in magnitude, raising the possibility that their importance

may be marginal relative to changes based on season or life history stage. While this study demonstrates the potential for dietary fatty acids, dietary anthocyanins, and training to produce differences among individuals, future studies may find it useful to investigate the effects of these factors within the broader regulatory context faced by wild songbirds.

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Tables

Table 1. Ingredients and fatty acid composition of semisynthetic diets used in this study.

Ingredients	% of Dry Mass	Oil Mixture (%)	
		High 18:2n6	Low 18:2n6
Agar ^a	3.19		
Casein ^a	19.12		
Cellulose ^a	4.97		
D-glucose ^b	39.18		
Amino Acid Mix ^c	2.68		
Salt Mix ^d	4.78		
Vitamin Mix ^d	0.38		
Elderberry Powder ^e	0.42		
Mealworms ^f	6.16		
Plant oils ^g	19.12		
Fatty Acid ^h :			
12:0		0.24	0.21
14:0		0.38	0.91
16:0		8.65	29.64
16:1n7		0.3	0.41
18:0		2.83	3.9
18:1n9		45.2	42.76
18:2n6		31.87	13.86
18:3n3		4.05	2.68
20:1n9		0.55	0.4
20:4n6		0	0
22:6n3		0.15	0.11
24:1		0.1	0.08

^a U.S. Biomedical Corp., Cleveland, OH; ^b Fisher Scientific, Waltham, MA; ^c Assembled after Murphy and King (1982) from individual amino acids supplied by Fisher Scientific; ^d MP Biomedicals, Santa Ana, CA; ^e High antioxidant diets only: Artemis International, Fort Wayne, IN; ^f Freeze dried: Exotic Nutrition, Newport News, VA; ^g low 18:2n6 diet: canola oil and palm oil, high 18:n6 diet: canola oil and sunflower oil, supplied by Jedwards International, Braintree, MA; ^h Diet fatty acid concentrations (percent by mass) was measured by gas chromatography in lipids extracted from the diets. Only the twelve most concentrated fatty acids are listed.

Table 2. Parameter estimates and Wald statistics for final models of flight muscle soluble protein concentration, CPT activity, CS activity, LDH activity, and HOAD activity. Trained birds fed the 13% 18:2n6 and high anthocyanin diet are the reference group for all models. Significant p-values are in bold.

Coefficients:	Estimate	SE	<i>t</i> - value	<i>P</i>
<u>Soluble Protein</u>				
Intercept	-482.1392	204.7227	-2.355	0.0207
Date	2.1014	0.6369	3.300	0.0014
Dietary FA	29.7065	22.2790	1.333	0.1858
Dietary AOX	-4.9034	22.3977	-0.219	0.8272
Training	-6.3271	22.2725	-0.284	0.7770
$R^2 = 0.138, df = 89$				
<u>CPT Activity</u>				
Intercept	0.3584	0.6676	0.537	0.5928
Date	0.0039	0.0021	1.836	0.0698
Soluble Protein	0.0011	0.0005	2.157	0.0338
Dietary FA	0.2579	0.1479	1.744	0.0847
Dietary AOX	-0.0566	0.0709	-0.798	0.4270
Training	-0.2289	0.0729	-3.149	0.0023
Soluble Protein : Dietary FA	-0.0015	0.0007	-2.286	0.0247
$R^2 = 0.236, df = 87$				
<u>CS Activity</u>				
Intercept	17.7864	40.4598	0.440	0.6613
Body Mass	0.7138	0.3244	2.201	0.0304
Date	0.0744	0.0879	0.846	0.4001
Soluble Protein	0.0420	0.0202	2.084	0.0401
Dietary FA	19.8003	5.9069	3.352	0.0012
Dietary AOX	-2.7343	2.8748	-0.951	0.3442
Training	-0.3869	3.1506	-0.123	0.9026
Soluble Protein : Dietary FA	-0.0822	0.0262	-3.132	0.0024
$R^2 = 0.194, df = 86$				
<u>LDH Activity</u>				
Intercept	-72.2454	198.4428	-0.364	0.7167
Date	0.9338	0.6472	1.443	0.1528

Soluble Protein	0.3536	0.2153	1.642	0.1043
Dietary FA	164.3125	62.9789	2.609	0.0107
Dietary AOX	103.9995	58.7329	1.771	0.0802
Training	-2.1888	21.4470	-0.102	0.9190
Soluble Protein : Dietary FA	-0.6736	0.2737	-2.462	0.0159
Soluble Protein : Dietary AOX	-0.4610	0.2852	-1.616	0.1098
Dietary FA : Dietary AOX	-219.1573	85.7707	-2.555	0.0124
Soluble Protein : Dietary FA : Dietary AOX	0.9108	0.3809	2.391	0.0190
$R^2 = 0.127, df = 84$				
<u>HOAD Activity</u>				
Intercept	-2.6776	6.0453	-0.443	0.6597
Date	-0.0017	0.0030	-0.566	0.5730
Soluble Protein	0.0475	0.0193	2.457	0.0169
Dietary FA	0.9417	0.6446	1.461	0.1487
Dietary AOX	0.5128	0.6419	0.799	0.4270
Training	-1.0416	0.6384	-1.632	0.1066
$R^2 = 0.110, df = 88$				

Table 3. Parameter estimates and Wald statistics for final models of leg muscle soluble protein concentration, CPT activity, CS activity, LDH activity, and HOAD activity. Trained birds fed the 13% 18:2n6 and high anthocyanin diet are the reference group for all models. Significant p-values are in bold.

Coefficients:	Estimate	SE	<i>t</i> - value	<i>P</i>
<u>Soluble Protein</u>				
Intercept	36.3225	137.4005	0.264	0.7921
Date	0.1994	0.4277	0.466	0.6422
Dietary FA	0.8378	14.7101	0.057	0.9547
Dietary AOX	32.2569	14.9102	2.163	0.0333
Training	37.1813	14.7796	2.516	0.0137
$R^2 = 0.123, df = 86$				
<u>CPT Activity</u>				
Intercept	1.3879	0.4515	3.074	0.0029
Date	-0.0001	0.0013	-0.018	0.9860
Soluble Protein	0.0005	0.0005	1.003	0.3190
Dietary FA	-0.2029	0.0949	-2.138	0.0354
Dietary AOX	-0.0295	0.0463	-0.636	0.5264
Training	-0.0961	0.0460	-2.092	0.0395
Wing Chord	-0.0038	0.0017	-2.254	0.0268
Soluble Protein : Dietary FA	0.0013	0.0006	2.027	0.0458
$R^2 = 0.164, df = 83$				
<u>CS Activity</u>				
Intercept	74.1489	26.3826	2.811	0.0062
Date	-0.0042	0.0754	-0.055	0.9561
Soluble Protein	0.0247	0.0292	0.845	0.4004
Dietary FA	-9.2082	5.5461	-1.660	0.1006
Dietary AOX	-0.5218	27.0686	-0.193	0.8476
Training	-4.3097	2.6855	-1.605	0.1123
Wing Chord	-0.2587	0.0982	-2.633	0.0101
Soluble Protein : Dietary FA	0.0708	0.0364	1.944	0.0553
$R^2 = 0.216, df = 83$				
<u>LDH Activity</u>				
Intercept	148.5661	174.5834	0.851	0.3973

Date	0.1516	0.5388	0.281	0.7791
Soluble Protein	0.8727	0.3065	2.847	0.0056
Dietary FA	74.9292	52.5432	1.426	0.1577
Dietary AOX	90.3228	61.7551	1.463	0.1474
Training	-4.7371	19.4013	-0.244	0.8077
Soluble Protein : Dietary FA	-0.8484	0.3985	-2.129	0.0363
Soluble Protein : Training	-1.0500	0.4193	-2.504	0.0143
Dietary FA : Training	-247.678	81.4473	-3.041	0.0032
Soluble Protein : Dietary FA : Training	1.9954	0.5401	3.695	0.0004
$R^2 = 0.264, df = 81$				
<u>HOAD Activity</u>				
Intercept	6.4305	2.3247	2.766	0.0070
Date	0.0018	0.0013	1.426	0.1576
Soluble Protein	-0.0123	0.0072	-1.706	0.0917
Dietary FA	-9.6646	3.1802	-3.039	0.0032
Dietary AOX	0.0779	0.1723	0.453	0.6522
Training	-0.3702	0.1784	-2.075	0.0411
Date : Dietary AOX	0.0305	0.0099	3.073	0.0029
$R^2 = 0.154, df = 84$				

Table 4. Parameter estimates and Wald statistics for final models of flight muscle CS expression and FAT/CD36 expression. Trained birds fed the 13% 18:2n6 and high anthocyanin diet are the reference group for all models. Significant p-values are in bold.

Coefficients:	Estimate	SE	<i>t</i> - value	<i>P</i>
<u>CS Expression</u>				
Intercept	7.4988	3.1779	2.360	0.0313
Body Mass	-0.0718	0.0280	-2.561	0.0209
Date	0.0061	0.0080	0.761	0.4576
Dietary FA	-0.0105	0.2186	-0.048	0.9621
Dietary AOX	0.6183	0.3381	1.829	0.0862
Training	0.1087	0.2248	0.483	0.6354
$R^2 = 0.453, df = 17$				
<u>FAT/CD36 Expression</u>				
Intercept	1.1307	0.4980	2.271	0.0424
Body Mass	0.0044	0.0034	1.284	0.2232
Date	-0.0037	0.0014	-2.535	0.0262
Dietary FA	-0.5444	0.6581	-0.827	0.4242
Dietary AOX	-0.0368	0.0370	-0.993	0.3402
Training	-1.5818	0.5909	-2.677	0.0202
Date : Dietary FA	0.0018	0.0020	0.885	0.3935
Date : Training	0.0048	0.0018	2.638	0.0217
Dietary FA : Training	2.1333	0.9233	2.310	0.0394
Date : Dietary FA : Training	-0.0066	0.0028	-2.327	0.0383
$R^2 = 0.758, df = 13$				

Table 5. Correlation coefficients for relationships among enzyme activities and expression in the pectoral muscle of European Starlings. Asterisks denote significance, with * indicating $P < 0.05$ and ** indicating $P < 0.01$.

	FAT/CD36 Expression	CPT Activity	CS Activity	HOAD Activity	LDH Activity
CS Expression ($N = 23$)	-0.44	-0.47	-0.43	-0.16	-0.06
FAT/CD36 Expression ($N = 23$)		0.11	0.11	0.00	-0.11
CPT Activity ($N = 94$)			0.51**	0.33*	0.13
CS Activity ($N = 94$)				0.04	0.04
HOAD Activity ($N = 94$)					0.15

Table 6. Correlation coefficients for relationships among enzyme activities in the leg muscle of European Starlings. Asterisks denote significance, with * indicating $P < 0.05$ and ** indicating $P < 0.01$.

	CS Activity	HOAD Activity	LDH Activity
CPT Activity ($N = 91$)	0.80**	0.44**	0.48**
CS Activity ($N = 91$)		0.26*	0.52**
HOAD Activity ($N = 91$)			0.19

Figures

Figure 1. The relationships between CPT activity (A, B), CS activity (C, D), LDH activity (E, F) and soluble protein concentration in the pectoral and leg muscle of European Starlings. Birds fed the 32% 18:2n6 diet (triangles, dashed lines) differed from those fed the 13% 18:2n6 diet (circles, solid lines). LDH activity was also

dependent on anthocyanin supplementation in pectoral muscle (E, light grey = unsupplemented) and flight training in the leg muscle (F, dark grey = untrained).

Figure 2. Flight training produced additive increases in the activity of CPT (A, B) in flight and leg muscle and HOAD (C) in the leg muscle of European Starlings.

Figure 3. The relationships between HOAD activity (A, B), soluble protein concentration (C, D), and Julian date in pectoral and leg muscle of European Starlings. Both HOAD activity and soluble protein concentration increased over time in the pectoral muscle, whereas in the leg muscle the effect of date on HOAD was dependent on dietary anthocyanins (light grey = unsupplemented) and soluble protein concentration additively decreased with training and anthocyanin supplementation (D, inset).

Figure 4. The expression of CS (A) and FAT/CD36 (B) in the pectoral muscle of European Starlings. CS concentration was negatively related to body mass, whereas FAT/CD36 exhibited dietary 18:2n6- (32% = dashed lines, 13% = solid lines) and flight training- (dark grey = untrained) specific changes over time.

MANUSCRIPT III:

Turnover of muscle lipids and response to exercise differs between neutral and polar
fractions in a model songbird, the Zebra Finch

by

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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 C₃ / C₄ diet shift experiment, with tissue samples taken at ten 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 $\delta^{13}\text{C}$ values via IRMS and estimated turnover in different fractions and treatment groups with nonlinear mixed effect regression. We found a significant difference between the mean retention times of neutral and polar lipids ($t_{119} = -2.22$, $P=0.028$), with polar lipids $\tau=11.80\pm1.28$ days) having shorter retention times than neutral lipids $\tau=19.47\pm3.22$ 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.

Keywords

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). Conversely, 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 for 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 due to 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 three-fold differences 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 from 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 (NL, e.g. triacylglycerols) and polar lipids (PL, e.g.

phospholipids) (Stevens, 2004). Given the broad separation of functional roles for these fractions, with NL primarily used in energy metabolism and PL in membranes, we might expect turnover to differ between them. As NL 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, PL 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; 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 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 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 (2.2m x 1m x 2m). We maintained the room on a 12h:12h L:D 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 C₄ mixed seed diet ($-15.57 \pm 0.34\text{‰}$ bulk $\delta^{13}\text{C}$, Hagen #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 C₃ mixed seed diet ($-27.70 \pm 0.01\text{‰}$ bulk $\delta^{13}\text{C}$, Abbaseed #3700, Hillside, NJ USA) primarily composed of canarygrass (*Phalaris canariensis*) for the remainder of the experiment. We measured fat score on a 0 to 7 scale (no visible fat to complete coverage, respectively, adapted from Eck et al., 2011) and body mass to the nearest 0.1g weekly for the duration of the experiment to monitor bird health.

Experimental treatments and tissue samples

We began flight training two 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 hrs of total flying time each day, divided into two 1-hr sessions starting at 1100 hr and 1330 hr, for the full 10 week period. During flight training, birds flew as a flock around a 6m x 3m x 2m flight arena with perches in opposite corners and a 4m 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 7m 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 both exercised and control birds were without food for the same periods.

On days 0, 1, 2, 4, 8, 16, 33, 56, 120, and 256 (relative to the diet shift), we sacrificed 2-4 birds from each experimental group (1-2 birds of each sex) 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 C₄ 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; Bauchinger and McWilliams, 2010). On a given day, all birds to be sacrificed were placed in cloth bags immediately after flight training and held for less than 80 minutes before sacrifice. Birds were weighed to the nearest 0.1g, checked for fat score, decapitated, and then the pectoral muscles and other selected organs were removed within 10 minutes. Tissues samples were then rinsed in water, blotted dry, weighed to the nearest 0.1mg, 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 400mg of wet tissue was homogenized in 6mL of 2:1 chloroform:methanol with a high-speed stainless steel homogenizer (PowerGen 700, Fisher Scientific, Waltham, MA USA), centrifuged at 3000rpm for 15 minutes, and then aqueous solutes were separated by rinsing with 2ml of 0.25% KCl. The organic phase was carefully removed with a Pasteur pipette and transferred to a 4ml glass vial where it was dried under N₂ and resuspended in 200μL chloroform. The fatty acid/ chloroform solution was then transferred to Supelclean solid phase extraction columns (Supelco, LC-NH₂, 1mL aminopropyl bonding) conditioned with 2mL hexane, and NL, non-esterified fatty acid (NEFA), and PL fractions were sequentially eluted with 1.8mL 2:1 chloroform:isopropanol, 2mL 49:1 isopropyl ether:acetic acid, and 2mL methanol.

NL and PL fractions were collected in 4mL 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 N₂, resuspending in 1M acetyl chloride in methanol, and heating at 90°C for 2hrs. We ground diet samples with a Wiley mill (#20 sieve; Thomas Scientific, Swedesboro, NJ) 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 N₂, and the ¹³C enrichment of each fractionated sample was measured by continuous flow isotope ratio mass spectrometry 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 ¹³C enrichment of aliquots of ground bulk diet and the non-lipid remainder of diet lipid extractions to compare $\delta^{13}\text{C}$ discrimination between tissue lipids and different diet components. Carbon isotope ratios were expressed as a permil deviation ($\delta^{13}\text{C} \text{ ‰}$) from the reference standard Pee Dee Belemnite (PDB): $\delta^{13}\text{C} \text{ ‰} = [(R_{\text{sample}}/R_{\text{PDB}}) - 1] \times 1000$, where $R = {}^{13}\text{C} / {}^{12}\text{C}$. Replicate FAME $\delta^{13}\text{C}$ 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.

Carbon Turnover and Statistical Analyses

As expected, carbon isotopic values displayed a pattern of exponential decay over time, thus requiring nonlinear 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 $\delta^{13}\text{C}$ value of the sample at time t in ‰, y_∞ is the estimated asymptotic $\delta^{13}\text{C}$ value of the tissue when it has come into equilibrium with the second diet in ‰, a is the estimated difference between the $\delta^{13}\text{C}$ values of tissues at the time of the diet shift and at equilibrium with the second diet in ‰, 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 fit 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 nonlinear 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 nonlinear least squares regression to use as starting values for step two. In the second step, we then used those estimated starting

values for nonlinear mixed effect models that included fixed effects of experimental group and sex on mean retention time and a random effect to account for variable $\delta^{13}\text{C}$ starting points among birds at the time of the diet shift. Two-compartment models failed to converge when fitting all 5 parameters, but successfully converged when we replaced the y_{∞} parameter with average $\delta^{13}\text{C}$ 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 information criterion corrected for small sample size) values for one and two-compartment models for each lipid fraction and selected the nonlinear mixed effect model with greater support ($\Delta\text{AICc} > 2$, 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 fit 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 $\delta^{13}\text{C}$ 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 were on average greater than that for the PL fractions (figure 1, $t_{119} = -2.22$, $P = 0.0279$), with the NL fraction having a mean retention time of $\tau = 19.5 \pm 3.2$ days (mean $\pm$ SE) and the PL fraction having a mean retention time of $\tau = 11.8 \pm 1.3$ days. Mean carbon retention time in the PL fraction of the pectoral muscle was significantly smaller in exercised birds ($\tau = 10.5 \pm 1.4$ days) compared to control birds ($\tau = 12.7 \pm 1.7$ days, figures 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 IQR) in the exercised group, removal of which increased the estimate of mean retention time ($\tau = 11.2 \pm 1.2$ days) and made the effect 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 (figures 1 and 2, exercised group: $\tau = 22.4 \pm 7.1$ days, control group: $\tau = 17.8 \pm 5.2$ days, $t_{56} = 0.569$, $P = 0.571$). There was no significant effect of sex on carbon turnover in either fraction. The measured $\delta^{13}\text{C}$ values of both NL and PL

fractions at day 256 closely matched the $\delta^{13}\text{C}$ value of the lipids in the C₃ diet, in contrast to the poor match with the bulk diet and lipid-free diet (figure 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 $\delta^{13}\text{C}$ 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, 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 $\delta^{13}\text{C}$ values and fat score in the PL fraction (figure 3, $r_{65} = 0.30$, $P = 0.01$) but not the NL fraction ($r_{65} = 0.20$, $P = 0.11$). Similarly, there was a significant positive correlation between residual PL fraction $\delta^{13}\text{C}$ values and the proportion of NLs in pectoral muscle ($r_{60} = 0.30$, $P = 0.02$) but not residual NL fraction $\delta^{13}\text{C}$ values ($r_{60} = -0.03$, $P = 0.81$). In contrast, there was no significant relationship between residual $\delta^{13}\text{C}$ 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 respectively NL, control PL, and exercised PL fractions (figure 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 two weeks to three weeks small (10 – 20g) 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 due to a more localized effect of metabolic rate via oxidative damage (Boom et al., 1996; Samuni et al., 2000; Wong-ekkabut 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 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 PL 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 two hour 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) that was not part of our study of only 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 past studies 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 $\delta^{13}\text{C}$ 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 rates 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 the NL fraction, depending on exercise condition (figures 1-2).

We also found considerably more variation in our estimate of turnover for the NL fraction (SE = 3.2 days) than for the PL fraction (SE = 1.4 – 1.7 days, depending on exercise condition), making turnover slower and more variable in the NL fraction.

Given relatively constant starting and asymptotic $\delta^{13}\text{C}$ 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 $\delta^{13}\text{C}$ value at any given time point could be attributed to variation in turnover rates among

individual birds. Thus, residual $\delta^{13}\text{C}$ 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 $\delta^{13}\text{C}$ values in the polar fraction and fat score suggests that turnover in the polar fraction is relatively slower in fatter birds and relatively 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 Río 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, 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 relatively smaller than the NL pool due to its higher proportions of ω -3 and ω -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 due to its more polyunsaturated character (Guglielmo et al., 2002b; Klaiman et al., 2009; McCue et al., 2009). This hypothesis, however, is not mutually exclusive from the effect of pool size described above, so 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 study 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 the PL fraction to published values of protein turnover in the pectoral muscle in Zebra Finches ($\tau = 21 \pm 2.55$ 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 due to 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 & 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 both 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 C₃ 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; Wang et al., 2015), these values may prove useful for future studies of songbird diet.

Symbols and Abbreviations

‰: parts per thousand

$\delta^{13}\text{C}$: ^{13}C carbon isotope ratio

FA: fatty acid

FAME: fatty acid methyl ester

MUFA: monounsaturated fatty acid

NL: neutral lipid

PL: polar lipid

PUFA: polyunsaturated fatty acid

SFA: saturated fatty acid

τ : mean retention time

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Tables

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

Fraction and Model	# Parameters	AICc	ΔAICc	Weight	Cumulative Weight	Log Likelihood
<u><i>Neutral Lipids</i></u>						
One Compartment	8	238.37	0.0	1	1	-109.90
Two Compartment	12	249.01	10.7	0	1	-109.53
<u><i>Polar Lipids</i></u>						
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

Table 2. Estimated tissue – diet discrimination factors ($\Delta^{13}\text{C}$) for bulk diet, lipid-extracted diet, and dietary lipids. Estimates are presented as means $\pm$ SE.

	$\delta^{13}\text{C}$	$\Delta^{13}\text{C}$ Neutral Lipids - Diet	$\Delta^{13}\text{C}$ Polar Lipids - Diet
Bulk Diet	-27.70 $\pm$ 0.01	3.41 $\pm$ 0.172	3.74 $\pm$ 0.169
Lipid-Extracted Diet	-26.89 $\pm$ 0.01	4.22 $\pm$ 0.172	4.55 $\pm$ 0.169
Dietary Lipids	-31.23 $\pm$ 0.01	-0.12 $\pm$ 0.172	0.21 $\pm$ 0.169

Table 3. Correlation coefficients between measures of fat storage and tissue composition of individual Zebra Finches ($N=65$).

	Fat Score	Percent Neutral	Percent Lipid
Percent Neutral	0.41		
Percent Lipid	0.32	0.46	
Adipose Mass	0.55	0.27	0.29

Figures

Figure 1. Mean carbon retention time (τ) in days for neutral and polar fractions of 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. Stars indicate significant differences between fractions or treatment groups. Turnover was significantly faster in the polar fraction than the neutral fraction ($T_{119} = -2.22$, $P = 0.0279$, two-tailed), and within the polar fraction turnover was significantly faster in the exercised group ($T_{56} = 2.37$, $P = 0.0212$, two-tailed).

Figure 2. Changes in $\delta^{13}\text{C}$ values following a C₄ to C₃ diet shift in the (A) neutral and (B) polar lipids of Zebra Finch ($N = 65$) pectoral muscles. Thin lines represent the exponential decay in $\delta^{13}\text{C}$ value for birds in the exercise (dashed) and control (solid) treatment groups. Solid heavy horizontal lines represent the two bulk diets, while dashed heavy horizontal lines represent fat and fat extracted components of the C₃ diet. The statistical outlier in the polar fraction is designated with a filled circle.

Figure 3. The relationship between residual $\delta^{13}\text{C}$ value and fat score in the (A) neutral and (B) polar lipids of Zebra Finch pectoral muscles. Residuals were calculated from the best-fit nonlinear regression model for each fraction and 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. 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.

MANUSCRIPT IV:

The dynamics of individual fatty acids in muscle fat stores and membranes of a songbird are affected by exercise and have functional and ecological importance

by

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Abstract

Although tissue fatty acid (FA) composition has been linked to whole-animal performance (e.g. aerobic endurance, metabolic rate, post-exercise 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 C₄ to C₃ diet shift experiment and compound-specific $\delta^{13}\text{C}$ 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 days in the polar fraction and 17.2 – 32.8 days in the neutral fraction, but was unexpectedly slow for the long-chain polyunsaturated FAs (LC-PUFAs) arachidonic acid (ARA, 20:4n6) and docosahexaenoic acid (DHA, 22:6n3) in the polar fraction, with 95% replacement in 64.9 – 136.5 days. Polar fraction LA and PA turnover was significantly faster in exercised birds (95% replacement in 8.5-13.3 days). Our results suggest that FA turnover in intramuscular lipid droplets is related to FA tissue concentrations and 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 fatty acid composition of diet affects energy metabolism of birds during migration.

Keywords

fatty acids, lipid droplet, membrane, metabolic rate, songbirds, turnover, CSIA

Introduction

Fatty acid (FA) composition of animal tissues is related to ecologically-relevant measures of whole-animal performance such as aerobic endurance, metabolic rate, post-exercise 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 fatty acids (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 depends 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 step towards predicting the function and fitness of individual animals in ecological contexts.

Although the FA composition of membranes and lipid stores play 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 which 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 availability of FAs (Ayre and Hulbert 1996; Pierce et al. 2004; McCue et al. 2009), which can vary both across individuals' diets (Frank, Karpovich & Barnes 2008) and within an individual's diet (Bairlein 1998; Smith *et al.* 2007; Klaiman, Price & Guglielmo 2009). Animals also exclusively rely on diet as a source of ω -3 and ω -6 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 time scales 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 eco-physiological changes in response to energetic challenges like migration or fasting (Wolf, Carleton, and Martinez del Rio 2009; Bauchinger and McWilliams 2010; Martínez del Rio and Carleton 2012; Ben-David and Flaherty 2012). With the increasing analysis of the isotopic composition of individual amino and fatty acids (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 non-functional, e.g. oxidative damage; Figure 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 linked directly to their tissue FA composition, growth, and metabolic rate (Price 2010; Pierce and McWilliams 2014; Martinez del Rio and McWilliams 2016; Twining et al. 2016b). 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 FAs (SFA) 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 ($\Delta^{13}\text{C}$), 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 (2.2m x 1m x 2m) with a 12h:12h L:D light schedule and 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 periods of daily flight training described below. During the ~3-month equilibration phase of the experiment (Days -90 to 0), we fed birds a C₄ mixed seed diet with a bulk $\delta^{13}\text{C}$ of $-15.6 \pm 0.3\text{‰}$ (Hagen #B2405, Mansfield, MA USA) primarily composed of millet (*Pennisetum glaucum*, Table S1). On Day 0 we switched birds to a C₃ mixed seed diet with the much lower bulk $\delta^{13}\text{C}$ of $-27.7 \pm 0.1\text{‰}$ (Abbaseed #3700, Hillside, NJ USA) primarily composed of canarygrass (*Phalaris canariensis*, Table 1) 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 two hours of flight training daily for ten weeks, from day -14 until day 56. During flight training a handler prompted birds to fly as a flock clockwise around a 6m x 3m x 2m flight arena, between perches in opposite corners. This resulted in approximately 8.4 km of short-burst flights per day, which is ~3X 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 two-hour exercise period each day from birds in the control group, which remained in their aviaries throughout.

Tissue Sampling and Lipid Extraction

We euthanized 2–4 birds from each experimental group (1–2 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 minutes before sacrifice. Birds were weighed to the nearest 0.1g, checked for fat score, decapitated, and the pectoralis muscles and other selected organs were removed within 10 minutes. Tissue samples were then rinsed in water, blotted dry, weighed to the nearest 0.1mg, 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 only been fed the C₄-based diet. We concentrated sampling in the days immediately following the diet shift to capture the period of expected greatest change in $\delta^{13}\text{C}$ values. Samples on Days 120 and 256 were included to allow robust fitting of exponential decay models (see below; Bauchinger and McWilliams 2009; Bauchinger and McWilliams 2010). All sacrifice and tissue sampling procedures were approved by the URI 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 400mg of wet tissue was homogenized in 6mL of 2:1 chloroform:methanol with a high-speed stainless steel homogenizer (PowerGen 700, Fisher Scientific, Waltham, MA USA),

centrifuged at 3000rpm for 15 minutes, and then aqueous solutes were separated by rinsing with 0.25% KCl. The organic phase was transferred to a 4ml glass vial by Pasteur pipette, dried under N₂, and resuspended in chloroform. Neutral lipid, non-esterified fatty acid (NEFA), and polar fractions were separated in solid phase extraction columns (Supelco, LC-NH₂, 1mL aminopropyl bonding) with sequential elutions of 2:1 chloroform:isopropanol, 49:1 isopropyl ether:acetic acid, and methanol. Neutral and polar fractions were collected in 4mL vials while the NEFA fraction was discarded. Fractionated samples were then esterified into Fatty Acid Methyl Esters (FAMES) by heating at 90°C for 2hrs in 1M acetyl chloride in methanol.

Fatty Acid 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 μ l aliquots of sample FAMES (1mg/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 USA) 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 $\delta^{13}\text{C}$ 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, Waltham, MA USA) at the University of New Mexico Center for Stable Isotopes (Albuquerque, NM). Sample FAMES were suspended in hexane at a concentration of 0.12mg/ml for neutral fraction samples and 0.20mg/ml for polar fraction samples, and a 1µl 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, Elysian, MN USA) of known $\delta^{13}\text{C}$ that had been methylated alongside unknown samples in the same batch, and used the offset between measured and known $\delta^{13}\text{C}$ values for this standard to correct sample $\delta^{13}\text{C}$ 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): $\delta^{13}\text{C} \text{ ‰} = [(R_{\text{sample}}/ R_{\text{standard}}) - 1] \times 1000$, where $R = ^{13}\text{C} / ^{12}\text{C}$ of the sample and 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 (v3.2.5, R Core Team, Vienna, Austria). 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: $y_t = y_{\infty} + (y_{\infty} - y_0)e^{(-\frac{t}{\tau})}$ to model changes in FA carbon isotope values over time, where y_t is the sample $\delta^{13}\text{C}$ of a given FA at time t in ‰, y_{∞} is the estimated asymptotic $\delta^{13}\text{C}$

value of the FA when it has come into equilibrium with the second diet in ‰, y_0 is the estimated $\delta^{13}\text{C}$ value of the FA at the time of the diet shift in ‰, 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 Río 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: nlme) 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, Cooper-Mullin, and McWilliams 2018). We calculated discrimination factors ($\Delta^{13}\text{C}_{\text{tissue-diet}}$) for individual FAs by subtracting C_3 diet FA $\delta^{13}\text{C}$ values from the estimated asymptotic $\delta^{13}\text{C}$ values produced by the mixed models for individual FAs described above.

Results

Fatty Acid Composition

Many of the same FAs were present in both the neutral and polar fractions of lipids extracted from our flight muscle samples (Figure 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. Fatty acid 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 (Figure 3), 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 days.

Carbon Isotopic Turnover of Fatty Acids

We measured $\delta^{13}\text{C}$ values and estimated turnover for four FAs in both the neutral and polar fractions (16:0, 18:0, 18:1n9, and 18:2n6), and two additional FAs (20:4n6 and 22:6n3) in the polar fraction (Figures 4, 5, and 6). Turnover in the polar fraction was fastest for 18:2n6 ($\tau = 4.1$ days, ± 0.4 ; mean $\pm$ 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 $\pm$ 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 days.

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 ($\tau_{\text{exercised}} = 3.7 \pm 0.8$, $\tau_{\text{control}} = 5.5 \pm 0.7$; $P = 0.029$, $T_{56} = -2.245$, figure 4a) 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 (figure 5b). 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 $\delta^{13}\text{C}$ values and diet for four FAs: 16:0, 18:0, 18:1n9, and 18:2n6 (Table 2).

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 ($\Delta^{13}\text{C} = 1.4 \pm 0.3$; 95% CI: 0.81 – 1.99), while the $\delta^{13}\text{C}$ 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 ($\Delta^{13}\text{C} = 1.0 \pm 0.5$; 95% CI: -0.07 – 2.07).

Discussion

Fatty Acid Composition

The FA profiles of muscle lipids in our zebra finches were largely consistent with previous studies on other songbirds (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 (Figure 2). Saturated (16:0) and monounsaturated FAs (18:1n9) 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 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 (Figure 3). This change may have been driven by a probable increase in dietary 18:1n9 (C₄ diet: 24.3% vs C₃ diet: 29.8%; Table 1). However, a shift of similar magnitude in dietary availability of 16:0 (C₄: 17.0% vs C₃: 11.4%) did not cause a similar change in bird tissue. Alternately, the slightly elevated protein content of our C₃ diet (15%) relative to C₄ 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.

Fatty Acid 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 (Figure 1, Table 3). First, all else being equal, measured turnover will be dependent upon 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 non-lipid 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 3). Second, the higher dietary concentrations of 18:2n6, 18:1n9, and 16:0 (Table 1) could result in more rapid dilution of those FA pools in muscle tissue (Table 3), 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 3). 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 3). 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 3). 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 sedentary birds if either of these mechanisms is driving FA turnover (Table 3).

Of these factors, turnover in the neutral fraction was most consistent with predictions based on tissue concentration (Table 3). 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 non-muscular 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 3). The slower turnover observed in 20:4n6 and 22:6n3 was only consistent with the predictions of diet concentration and suggests that (1) songbirds 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 3). 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 the neutral fraction for 18:2n6, 16:0, and most notably 18:1n9 (Figures 4, 5, and 6), 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 (Figure 2) and elevated catabolism (as evidenced by exercise effects; Figure 5) likely explain the faster turnover of the polar fraction.

Fatty Acid Tissue-Diet $\delta^{13}\text{C}$ 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 $\delta^{13}\text{C}$ discrimination relative to those in diet (Table 2): 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 $\delta^{13}\text{C}$ 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 $\delta^{13}\text{C}$ 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 $\delta^{13}\text{C}$ 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 they undertake, whereas for 18:2n6 it likely suggests selective oxidation or further metabolic processing in some individuals. Overall, these results indicate 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 one week; 50% of these FAs turn over in 3-4 days and 95% turn over in <18 days. 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 days, 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 days. The long residence times and corresponding low demand for these PUFAs suggest that their low dietary concentrations is 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; Käkälä et al. 2009; Twining et al. 2016a). 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 (Martinez del Rio and McWilliams 2016; Twining et al.

2016b). 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 3.6mg of neutral and 2.0mg 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 0.88mg of 18:2n6 per gram of muscle per day to maintain their FA composition; this requires a dietary FA concentration of at least 1.3mg/g (assuming total food intake of 1.5g 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*, 15.9mg/g in *Parthenocissus quinquefolia* (Zygadlo *et al.* 1995; Smith *et al.* 2007; McCue *et al.* 2009; Patterson & Magnuson 2014; Pierce & 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.

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Tables

Table 1. Bulk nutritional information for the seed diets used in this experiment

(Hagen #B2405, Mansfield, MA USA; Abbaseed #3700, Hillside, NJ USA) and fatty acid composition (% by mass) of their primary seed ingredients, millet (*Pennisetum glaucum*) and canarygrass (*Phalaris canariensis*) respectively. Millet constituted 78% of the biomass consumed by birds on the C₄ diet, while canarygrass constituted 90% of the biomass consumed by birds on the C₃ diet (Carter, unpublished data).

Diet	C ₄ : Hagen B2405	C ₃ : Abbaseed 3700
Protein (%)	11	15
Fat (%)	5	6
Fiber (%)	10	12
Moisture (%)	12	14
Ash (%)	5.5	6
Primary Seed	Millet	Canarygrass
Fatty Acid ^a		
14:0	0.1 ±0.0	0.2 ±0.1
16:0	17.0 ±5.3	11.4 ±0.3
16:1	0.5 ±0.4	0.2 ±0
18:0	3.7 ±1.6	1.2 ±0.1
18:1n9	24.3 ±3.5	29.8 ±0.3
18:2n6	50.5 ±10.2	53.2 ±1.0
18:3n3	3.0 ±2.1	2.4 ±0.1
20:0	0.8 ±0.4	0.1 ±0.1
20:1	0.3 ±0.3	1.0 ±0.1
22:0	0.4 ±0.1	0.1 ±0.1

^a Millet fatty acid composition averaged from Sawaya et al. (1984), Kumar et al. (2008), and McCue et al. (2009). Canarygrass fatty acid composition averaged from Abdel-Aal (1997), Patterson and Magnuson (2014) and CDCS Nutritional Factsheet (2016).

Table 2. Equilibrium $\delta^{13}\text{C}$ values and estimated tissue-diet discrimination factors ($\Delta^{13}\text{C}$) for neutral and polar fractions of pectoral muscle lipids. Values are presented as mean $\pm$ SE. The shaded discrimination value highlights a significant positive discrimination factor for 16:0 in the polar fraction. Where present, different letters indicate significant differences in equilibrium $\delta^{13}\text{C}$ enrichment.

$\delta^{13}\text{C}$ (‰)	16:0	18:0	18:1n9	18:2n6
C ₃ Diet	-31.1 $\pm$ 0.2 ^A	-30.4 $\pm$ 0.4	-29.5 $\pm$ 0.1	-30.1 $\pm$ 0.3
Neutral	-30.1 $\pm$ 0.5 ^{AB}	-29.4 $\pm$ 0.5	-29.6 $\pm$ 0.6	-30.3 $\pm$ 0.5
Polar	-29.7 $\pm$ 0.2 ^B	-30.7 $\pm$ 0.4	-29.8 $\pm$ 0.4	-30.7 $\pm$ 0.4
$\Delta^{13}\text{C}$				
Neutral	1.0 $\pm$ 0.5	1.0 $\pm$ 0.7	-0.1 $\pm$ 0.6	-0.2 $\pm$ 0.6
Polar	1.4 $\pm$ 0.3	-0.3 $\pm$ 0.6	-0.3 $\pm$ 0.4	-0.6 $\pm$ 0.5

Table 3. Predicted and observed rank ordering of fatty acid turnover rates and presence of exercise effects. 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 text for rationale). Ranks are from fastest to slowest.

		Turnover Rate	Observed	Predicted ranking based on different influences			
				Adjusted Tissue Concentration	Diet Concentration	Oxidative Damage	Functional Catabolism
FA Fraction	<i>Neutral</i>	Fastest ↓ Slowest	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
			18:1n9	18:1n9	18:0	18:1n9	18:0
<i>Polar</i>		Fastest ↓ Slowest	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
Exercise Effects?			20:4n6	18:0	20:4n6	18:0	18:0
			22:6n3	18:1n9	22:6n3	18:1n9	18:1n9
			Neutral: N, Polar: Y	N	N	Y	Y

Figures

Figure 1. Factors hypothesized to influence fatty acid turnover in animal tissues. (A1) 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. (A2) Larger tissue pools take longer to dilute or to be catabolized, resulting in a negative correlation between pool size and turnover rate. (A3) 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.

Figure 2. Concentrations of the ten 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 $\pm$ SE.

Figure 3. Changes in the concentration of 18:1n9, measured as mass percent ($\pm$ SE), over time following a shift from a C₄ to C₃-based diet in the neutral fraction of zebra finch flight muscle lipids. The line depicts the best fit first-order kinetic equation ($y_t = 30.74 - 24.90 * e^{(-0.039t)}$) describing the trend.

Figure 4. Mean $\delta^{13}\text{C}$ values ($\pm$ SE) of FAs in the (A) polar and (B) neutral fractions of zebra finch flight muscle lipids, sampled at ten time points following a shift from a C_4 - to 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 $\delta^{13}\text{C}$ values soon after the diet shift.

Figure 5. Estimated mean retention times (τ , days) of FAs in the (A) polar and (B) neutral 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 stars indicate significant differences between exercised and control groups.

Figure 6. Mean $\delta^{13}\text{C}$ values ($\pm$ SE) of FAs in the polar (A, B, and C) and neutral (D and E) fractions of zebra finch flight muscle lipids, sampled at ten time points following a shift from a C_4 - to C_3 -based diet. Curves represent the estimates of first-order kinetic models of ^{13}C turnover for each FA. 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 $\delta^{13}\text{C}$ values soon after the diet shift.

MANUSCRIPT V:

The importance of isotopic turnover for understanding key aspects of animal ecology
and nutrition

by

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Abstract

Stable isotope-based methods have proved to be immensely valuable for ecological studies ranging in focus from animal movements to species interactions and community structure. Nevertheless, the use of these methods is dependent on assumptions about the incorporation and turnover of isotopes within animal tissues, which are oftentimes not explicitly acknowledged and vetted. Thus, the purpose of this review is to provide an overview of the estimation of stable isotope turnover rates in animals and to highlight the importance of these estimates for ecological studies. Specifically, we discuss 1) the factors that contribute to variation in turnover among individuals and across species that influences the use of stable isotopes for diet reconstructions, 2) the differences in turnover among tissues that underlie so-called ‘isotopic clocks’ that are used to estimate the timing of dietary shifts, and 3) the use of turnover rates to estimate nutritional requirements and reconstruct histories of nutritional stress from tissue isotope signatures. As we discuss these topics, we highlight recent works that have effectively used estimates of turnover to design and execute informative ecological studies. Our concluding remarks suggest several steps that will improve our understanding of isotopic turnover and support its integration into a wider range of ecological studies.

Keywords: turnover rate, stable isotope analysis, diet reconstruction, isotopic clock, nutritional status

Introduction

For more than thirty years, stable isotopes have been used as tools to address a wide range of questions in ecology, from elucidating key aspects of physiology and nutrition to tracking the movement of animals and defining the structure of biological communities [1–3]. The value of stable isotope-based tools has been demonstrated repeatedly throughout this period, leading to an exponential increase in their use: a web of knowledge search for the topic “stable isotope*” yields fewer than one hundred results from the 1970s and more than four thousand in each of the past three years. However, the successful use of stable isotope-based tools by ecologists requires careful consideration of the fundamental processes whereby stable isotopes are metabolized and incorporated into the animals and plants being studied [4–7]. In particular, the assimilation of dietary nutrients into the organism [2,8,9], the routing and fractionation of assimilated nutrients within the organism [6,10,11], and the rate of isotopic incorporation or turnover [12–15] all have substantial influence on the results and interpretation of isotope-based ecological studies. Accordingly, considerable progress has been made in testing assumptions about assimilation, routing, and isotopic turnover in lab settings, a necessary step to ensure that those processes are properly accounted for in ecological studies [4,6]. Nevertheless, in spite of the work estimating the turnover rates of tissues in a wide range of taxa [16,17], the importance of turnover processes to whole-animal metabolism and isotopic signatures is often not explicitly recognized in studies using isotope-based tools. Therefore, the purpose of this review is to 1) illustrate how stable isotopes can be effectively used to estimate the turnover rates of animal tissues and key compounds

within tissues, and 2) discuss the use and importance of isotopic turnover estimates for ecological and nutritional studies, with a particular focus on how metabolic physiology provides the foundation for these applications. We also outline the potential future of isotopic turnover measurements and particular challenges that future studies may approach using this methodology.

How to measure turnover rates with stable isotopes: from elements to molecules

Estimating the turnover of a compound or tissue typically requires labeling the molecules of interest at one time and then tracking the concentration of those labels over time as the molecules are excreted, degraded, or converted into other forms and replaced with unlabeled molecules (Figure 1A). The isotopic turnover of a compound or tissue therefore involves labeling the constituent atoms of molecules of interest with traceable stable isotopes of the elements that make up the molecule.

Theoretically, any element could be labeled and tracked, but for practical reasons most studies of isotopic turnover have focused on carbon, nitrogen, and hydrogen using the ^{13}C , ^{15}N , and ^2H isotopes respectively. Turnover rates have also been estimated for the ^{34}S and ^{18}O isotopes of Sulfur and Oxygen, respectively, but estimates for these elements have been far less common [17–19].

The isotopic label used in a given study should be chosen to match the compounds of interest in that study (Figure 1B). Carbon, as the defining constituent, can be used to label all organic molecules, making it the most relevant element for the majority of isotopic turnover studies. Hydrogen and oxygen are also applicable to most organic molecules found in animal tissues and are also commonly used to

measure the turnover of body water, which underlies the estimation of energy expenditure with doubly labeled water [20–23] as well as body composition with deuterium [24,25]. In contrast, nitrogen is restricted to amino and nucleic acids and sulfur is primarily found in the amino acids cysteine and methionine, making ^{15}N and ^{34}S most applicable to measuring the turnover of proteinaceous tissue. Thus, for studies focused on the turnover of bulk tissues, ^{13}C will typically be the most straightforward label available.

Most studies, however, are interested in the turnover of more specific tissue components (e.g., proteins and fats, or amino acids and fatty acids), which requires either labeling those specific components or separating them prior to stable isotope analysis. Separating tissue samples into their macromolecular components can be readily achieved by isolating lipids and carbohydrates from protein components via lipid extraction [26–28] and cation exchange purification [29–31], respectively. Compounds of similar macromolecular classes can then often be separated by gas-chromatography [11,32–34]. Alternately, studies focused on protein turnover could label just that tissue component with ^{15}N or ^{34}S enriched amino acids. Even more specific labels can be created for any compound by positioning isotopically heavier atoms at specific positions within the molecule [35–37], although these may also require purification before analysis. In general, highly specialized labels will be most useful when the study is measuring the turnover of a small set of very specific compounds, and appears less beneficial as the scope of the study widens. For many ecological applications whole-tissue or macromolecular turnover will be sufficient,

whereas the turnover of specific compounds may be more important for nutritional and pharmacological studies.

Once an isotopic label has been chosen, the frequency, or enrichment, of that label needs to be manipulated, so the rate of change over time can be measured (Figure 1C). Typically, this will involve either enriching or depleting the tissue(s) with the label and then reversing the enrichment/depletion to ensure that a large enough change occurs to be accurately and precisely measured. Large scale manipulations are usually most readily accomplished with complete shifts in diet. For example, tissue ^{13}C concentration can be enriched by feeding animals diets based on C_4 plants or depleted with diets based on C_3 plants or enriched with marine diets and depleted with terrestrial diets [9,12,38–42]. Similarly, ^{15}N can be enriched by using animal protein in diets and depleted by using plant protein [9,39,43]. It may also be possible to manipulate tissue ^2H by sourcing diets from different locations along the geographic ^2H gradients [9,44,45]. Drinking water can also be spiked with ^2H or ^{18}O to produce whole-animal enrichment with those labels. For such large-scale diet manipulations, equilibrating animals with the initial diet is ideal to ensure all tissues have a consistent and predictable isotope value, but this may not always be possible for tissues and species with very slow turnover rates. For more specific labels, direct administration is often applied with a dose of labeled molecules into the digestive tract by gavage or directly into the bloodstream or tissue by injection.

Previous studies and reviews have discussed the collection of tissue samples and analysis of isotopic turnover data in great detail and are excellent resources for those designing experiments [19,46–48]. Briefly, several particularly important

considerations are the number and spacing of samples over time following manipulation of the isotopic label and the statistical model used to describe changes in isotopic enrichment. The goal of sampling is to precisely track changes in isotopic enrichment, so as many samples should be taken as possible. However, when the number of samples is limited, they should be concentrated in the period immediately following the diet shift or administration of the label (Figure 1D). This spacing, usually following a geometric pattern (e.g. 0, 1, 2, 4, 8, etc.), ensures that more samples are taken when changes in isotopic enrichment are occurring most rapidly. Changes in isotopic enrichment are typically described and turnover rates estimated using exponential decay models (Figure 1F), with the most commonly used being first order kinetic models of the form:

$$y_t = y_{\infty} + (y_{\infty} - y_0)e^{\frac{-t}{\tau}}$$

or

$$y_t = y_{\infty} + (y_{\infty} - y_0)e^{-\lambda t}$$

where y_t is the isotopic enrichment at time t , y_{∞} is enrichment at equilibrium with the second diet in ‰, y_0 is enrichment at the time of the diet shift in ‰, t is the time since the diet shift, and τ is the mean retention time of the isotope, which can be replaced with λ , the kinetic rate constant equal to $1/\tau$. Nevertheless, other options are possible, most notably multi-compartment models [46,49], and may be favored on either empirical grounds or for mechanistic reasons if it is known that multiple sources contribute to the isotopic makeup of a given tissue or pool of molecules. Another important consideration for studies focused on the turnover of specific compounds is the interconversion between different molecules. This interconversion can decouple

isotopic labels from their original compounds and should be accounted for by either isolating the compounds of interest during analysis or by correcting the measured isotopic enrichment of samples for the rate of conversion to other forms [11,50,51].

The dynamics of stable isotopes in organisms: a physiological foundation of ecological applications of stable isotopes

Once the turnover rate of a compound, tissue, or tissue component has been measured, it can then be used to make inferences about the anabolic and catabolic processes that, in sum, produced those changes in composition. Moreover, comparisons across tissues or individuals can further enhance our understanding of organismal physiology, from cell-level processes to whole-animal metabolism, which in turn provides the foundation for the use of stable isotopes in ecological studies. Here we discuss some of the important physiological findings of turnover rate studies including 1) differences among individuals with different metabolic rates and body masses, which informs the estimation of turnover rates by allometry, 2) repeatable differences among tissues, which underlie the use of isotopic clocks, and 3) the implications of turnover rates for nutrient supply and dietary requirements for nutrients and energy.

Considerations for isotopic diet reconstructions: variation in turnover among individuals

From the early studies on isotopic turnover the rate of energy metabolism was hypothesized to be a major determinant producing variation in tissue turnover rates across species [12,38,52]. The following thirty years of research on proteinaceous

tissue, however, illustrate a slow and gradual shift away from the importance of quantitative metabolism (energy) to the hypothesis that the quality of metabolism (structural turnover) produces the variation among individuals. Three studies mark a turning point along that shift from quantity of energy to quality of structure in that they performed experiments to test predictions based on the hypothesized positive association between energy metabolism and isotopic turnover in protein. The studies altered the energy use of birds through either manipulation of ambient temperature [53,54] or flight exercise [54,55] and measured isotopic incorporation into blood (and other tissues in one study, [54]). Despite clear expectations, all three studies confirmed that energy metabolism was not the primary driver of isotopic turnover. Carleton and Martinez del Rio [53] first suggested that protein turnover rather than energy metabolism was the main driver of isotopic turnover. The evidence from these three studies revealed that doubling of energy metabolism [53,54] as well as flying or not flying over extended time periods in a wind-tunnel [55] did not alter blood isotope turnover in three different songbird species. Direct support for protein turnover affecting isotopic turnover came from diet-switch studies of a songbird [56] and rats [57]. For example, Yellow-Vented Bulbuls (*Pycnonotus xanthopygos*) fed a low protein diet had slower rates of nitrogen incorporation into plasma and blood cells than birds fed a high protein diet [56]. Comparable results were obtained on rats for which isotopic turnover of carbon and nitrogen for various organs increased by 20% and 30%, respectively, when fed a diet enriched with protein compared to a standard diet [57]. This evidence suggests that rate of protein turnover rather than energy

metabolism primarily determines rate of tissue carbon turnover between species, although clearly more such studies on a broader variety of species are needed.

Although a consensus is forming that structural turnover rather than metabolic rate is the main driver of carbon isotope incorporation rates, there remains uncertainty, particularly for less studied isotopes, tissues, and taxa. Similar to the studies above, Storm-Suke et al. [58] found no effect of metabolic rate on the turnover of ^2H in the red blood cells of Japanese Quail (*Coturnix japonica*), but this remains one of the few studies to quantify deuterium turnover in animal tissue. In contrast, Colborne et al. [59] found opposing responses of ^{15}N and ^{13}C turnover in response to elevated temperature (and therefore metabolic rate) in Emerald Shiners (*Notropis atherinoides*), which could be attributed to the involvement of different metabolic pathways for carbon and nitrogen or to differences in metabolism between endotherms and ectotherms [60–62]. Finally, studies of lipid turnover have observed effects of energy expenditure more regularly than those focused on protein [63–65], suggesting that differences in the metabolism of macromolecules may entail different relationships with energy use. While these are all interesting patterns, they are the result of comparatively few studies and should be further investigated in the future.

Besides such variation in isotope turnover among individuals of the same body mass and species, allometry of isotope turnover across species seems evident although the range of species for which such turnover rates are available needs to be expanded. Establishing how turnover scales with body mass, its allometry, for many tissues would greatly expand our ability to predict isotope turnover for animals across a much broader range of body sizes, and then use this to better understand key aspects

of their ecology. Like so many other physiological and morphological features – including energy metabolism (for general review see [66,67]) – isotopic turnover appears to be related to body mass. The allometry of isotopic turnover was first evaluated for avian whole blood [53] and specifically for the cellular fraction of blood [68]. Subsequent meta-analyses provide estimates of isotopic incorporation rates at the level of the whole organism, for specific taxonomic groups and for specific organs [16,17]. For example, Thomas and Crowther [16] used body mass and body temperature to predict rates of isotopic incorporation into tissues of a broad suite of endotherms and ectotherms. They found that whole animal and muscle turnover rate that scaled with body mass to the power of -0.19. Vander Zanden and colleagues [17] also found that turnover rates of many tissues across a variety of taxonomic groups scaled allometrically and discussed the implications of such allometry for ecological studies of food webs and trophic relationships. Despite these recent meta-analyses, our current understanding of the allometry of isotopic turnover rates is far from complete and requires many more empirical studies of tissue turnover rate across a wider variety of species that differ broadly in body mass.

The physiological basis of isotopic clocks: variation in turnover among tissues

Ecologists are often interested in determining the timing of shifts in resource (diet) use in relation to the phenology and availability of the resource. The isotopic value of a tissue such as red blood cells and plasma can be used to estimate the timing of these diet shifts, as a so-called ‘isotopic clock’, given certain conditions: (a) the turnover rate of the sampled tissue(s) (e.g. red blood cells, plasma) must be known, (b)

resources must differ in their isotope value, (c) the resource shift should be relatively clean, meaning from one resource to another at a certain time, and (d) the timing of the tissue sampling must occur while carbon (or other elements) from the initial diet is still apparent in the tissue(s) and carbon from the new diet has adequate time to be incorporated [69]. Such conditions quite commonly occur, for example, in animals living in seasonal environments where seasonal changes in resources must be tracked by animals or the animals migrate to more benign environments with quite different resources. For example, Yellow-Rumped Warblers (*Dendroica coronata*) that inhabit forests in the northern reaches of North America during the breeding season then migrate south to the coasts - those warblers that migrate to the east coast of North America feed almost exclusively on myrtle berries which have a uniquely negative carbon value (-28‰) that is quite different than their summer resources [70]. If one knows the turnover rate of a given tissue(s), and resources differ in their isotopic composition, then the timing of the resource shift (or how long Yellow-Rumped Warblers have been on the coast consuming myrtle berries) can be determined. Podlesak et al. [70] non-destructively sampled blood and breath and feces and feathers from many Yellow-Rumped Warblers and used the known differences in turnover rate of these tissues to estimate within a few days the timing of each individual's arrival to coastal New England.

Very few studies have quantified the turnover rates of multiple tissues within the same individuals (for review see [68]), but a few generalizations seem to be emerging. First, the differences in turnover between tissues (Figure 2A) are orders of magnitude larger than that caused by any whole-animal change in energy or protein

metabolism. Second, although many more such studies are needed, the relative differences in turnover rate between organs seem consistent across at least the six species of birds that have been studied. Third, for those tissues that have been studied across a range of species of different body size, the turnover rate scales allometrically (see discussion in the previous section). Lastly, if such robust between-organ differences in turnover rate are confirmed with more studies, then this makes available a quite refined isotopic clock for estimating the timing of resource shifts in a wide variety of animals.

If energy metabolism does not explain variation in turnover rate within a tissue, as discussed above, it seems also unlikely that energy expenditure explains variation between tissues within individuals. The few studies that have measured turnover rate in a variety of tissues confirm that turnover rate differences between tissues cannot be explained by variation in energy expenditure of these tissues [71,72]. Splanchnic organs like liver and small intestine appear to have fast rates of isotopic incorporation while muscles have slow rates and internal organs like gizzard, kidney and heart arrange themselves in-between. It appears that some tissues like brain, bone and skin pose some issues in that they are either extremely slow in turnover rates and thus don't reach asymptotes in the respective studies or the lack of fit is a result of large variation in the determinant of the isotopic incorporation [54,68]. Protein synthesis and degradation of the respective organ more likely determine the rate of isotopic incorporation than the simple energy turnover of the respective organ as demonstrated through dietary manipulations (see above and [56,57,72]). Also, the study that employed manipulations of energy budget through

both temperature manipulation and manipulation of flight exercise with the goal to test for predicted associations between energy use and isotopic turnover (see section just above) sampled not only the blood but a variety of organs [54]. For example, flight and leg muscle represent tissues that undoubtedly differ in their use in response to thermoregulation or flight exercise and thus rate of energy transformation into physical work. However, flight muscle isotopic turnover did not differ between flight exercise treatment and control, a result comparable to that for the leg muscle, clearly not in use during flight. In contrast, temperature treatment resulted in differences in flight muscle carbon turnover with faster turnover under cold treatment compared to warm temperature, leg muscle again revealed no difference. Flight muscle is the site for shivering thermogenesis and thus higher energy use for contraction since birds do not use uncoupling proteins. Energy use in an organ may thus still be linked to its turnover, but clearly this is not always the case. The contributions of the different metabolic processes to the organ specific rate of isotopic turnover requires cleverly designed experiments that manipulate certain metabolic processes and determine how this affects turnover rate of multiple tissues.

Turnover and diet requirements

The isotopic turnover of a tissue in steady state involves both the removal of old molecules by catabolism, and their replacement with newer, isotopically distinct molecules [12,38,71]. Thus, estimates of catabolic turnover also represent the rate at which molecules need to be supplied to the tissue to maintain that steady state, which can then be used to make inferences about the supply of the compounds in question

(Figure 3A). For example, estimates of lean tissue turnover are also representative of the rate of protein synthesis, which is in turn related to energy expenditure and minimum rates of non-essential amino acid synthesis and the dietary intake of essential amino acids [73,74]. Studies that thoroughly consider the implications of turnover rates for energetic and nutritional status are relatively uncommon, but several have pursued and demonstrated the utility of this method. For example, Mizrahy et al. [75] used the protein turnover rates estimated by Bauchinger and McWilliams [68] to calculate the rate of protein synthesis, and therefore the minimum amount of energy required to rebuild lean tissue after a fast that simulated in-flight starvation in Blackcaps (*Sylvia atricapilla*). The contrast between that minimum requirement and the birds' actual intake of energy then informed their conclusion that water availability influenced the recovery of tissues after the fast by affecting digestive efficiency.

A second example is the study by Carter et al. [65], which estimated the turnover rates of fatty acids in the membranes and lipid droplets of Zebra Finch (*Taeniopygia guttata*) pectoralis muscle. Certain polyunsaturated fatty acids (PUFAs) are essential or conditionally essential nutrients for vertebrates [76–78], so Carter et al. were then able to calculate minimum daily requirements for the essential n6 fatty acid linoleic acid as well as minimum dietary concentrations. Although estimated requirements were higher on a per gram basis than for larger animals, the authors were able to conclude that many of the dietary items wild songbirds encounter would meet those minimum levels. In contrast, the very slow turnover of the long-chain PUFAs arachidonic acid and docosahexaenoic acid led Carter et al. to conclude that

very low dietary concentrations were required to maintain a steady composition of these functionally important fatty acids.

A final example is a 2016 study by Salini et al. [79], who measured the turnover of fatty acids in barramundi (Asian Seabass; *Lates calcarifer*). This study similarly found very low rates of turnover for long-chain PUFAs with correspondingly low maintenance requirements and concluded the demand for these fatty acids was largely driven by deposition in tissues. Because deposition depends on growth, the authors also concluded that dietary requirements would be highest in younger, faster growing individuals. Although the scope of these studies does not extend to a comprehensive range of tissue components and dietary nutrients, they do effectively demonstrate the utility of turnover rates for understanding the digestive physiology and nutrition of animals.

The use and importance of turnover data for isotopic studies of ecology and nutrition

Stable isotope analysis has become a vital tool for studies seeking to characterize the diets of animals and the timing of diet shifts, and subsequently to use that data to infer the structure of ecological communities [1,80–83]. However, the successful use of stable isotopes in these applications depends on full consideration of the physiology that underlies the incorporation of isotopes into tissues, represented in measurements of turnover rates of compounds or tissues. Here we discuss the influence of turnover rate measurements on diet reconstructions, isotopic clocks, and assessments of nutritional status in stable isotope studies.

Reconstructing diets from isotopic signatures

Perhaps the most common use of stable isotope analysis in ecology is to identify the dietary items and the proportions in which they are consumed and assimilated by animals. Such diet reconstructions require that potential dietary items possess distinct dietary signatures, whose influence on tissue isotope values can then be disentangled with Bayesian mixing models [2,7,84–86]. Differences in the isotopic signature of dietary items require differences in isotopic fractionation, which can result from physical processes (e.g. geographic ^2H gradients) [9,44,45,87], metabolic pathways (e.g. C_3 vs. C_4 plant $\delta^{13}\text{C}$) [9,12,42], trophic level (e.g. plant vs. consumer $\delta^{15}\text{N}$) [9,39,43], or biome (e.g. marine vs. terrestrial $\delta^{13}\text{C}$ and $\delta^{15}\text{N}$) [9,39–41]. Once diet has been established, that information can then be used to answer a wide range of ecological questions, including what resources are used by different species [13,88–90], where individuals move to acquire those resources [91–94], how energy and nutrients move among trophic levels [33,34,95,96], and how communities and biomes are linked by the flow of energy and nutrients [41,97–99]. The diversity and novelty of these applications contribute to the great popularity of stable isotope-based diet reconstruction methods.

However, the isotopic signature of tissues changes over time following diet shifts (Figure 2B) and differences in turnover among tissues and individuals mean that different samples may integrate the effect of diet over shorter or longer periods of time [1,15,46]. Integrated isotopic signatures impede the estimation of diet for a specific time point, while variability among samples can reduce the precision of diet reconstructions. The most common way to account for these changes in composition

over time is to assume that the sampled tissues are in equilibrium with the diet and so consequently there are no changes over time to disrupt diet reconstruction [16,17]. It is critical for the reliability of dietary studies that these assumptions be validated, ideally with actual estimates of turnover rates in the study species, but with estimates from similar species or inferred by allometry if species-specific estimates are unavailable and impractical to obtain. It is particularly useful to consider the turnover rates of tissues in the focal species during the design phase of the study, as samples can then be chosen to represent diet at different time points in the past [1,46,100,101]. Tissues with fast turnover rates (e.g. plasma, liver) will represent the short-term diet whereas tissues with slow turnover rates (e.g. muscle, bone) will be more influenced by long-term diet, and inert tissues (e.g. hair, feather) will represent diet during the period when it was grown. Thus, considering the turnover rates of animal tissues not only improves the quality of diet reconstructions, it also expands the questions to which stable isotope analyses can be applied.

A few interesting examples will illustrate how knowledge of turnover rates of different tissues from the same individuals helps to document resource use over time in animals. A recent study by Marques et al. [102] used stable isotope analysis of liver, muscle, and feather samples to reconstruct the diets of Magellanic Penguins (*Spheniscus magellanicus*) at several points throughout their annual cycle. Overall, they found high reliance on the Argentine Anchovy (*Engraulis anchoita*), but they also were able to reveal interesting changes over the course of the annual cycle and differences among adults and juveniles. Specifically, anchovy consumption was highest during the migratory/early wintering period, as indicated by muscle, but diets

were more diverse in the mid and late winter, as indicated by the liver, with notable consumption of the Silverside (*Odontesthes argentinensis*) and São Paulo Squid (*Doryteuthis sanpaulensis*). Similarly, diet during the nestling/post-breeding period, as indicated by feathers, was also more diverse, with substantial contributions by the White Shrimp (*Peisos petrunkevitchi*). Interestingly, diets diverged between adults and juveniles least during the migratory/early wintering period, suggesting that adults use different resources than juveniles except when dietary options are limited. This result again highlights the importance of Argentine Anchovy, their primary food source during that period, to Magellanic Penguins.

A second example of the effective selection of tissues on the basis of turnover, is the recent study by Gómez et al. [103], in which they estimated changes in the trophic position of a migratory songbird, the Gray-Cheeked Thrush (*Catharus minimus*), over its annual cycle. The authors collected whole-blood samples to represent diet shortly preceding capture during spring migration, claw samples to represent diet on wintering grounds, and feather samples to represent diet during the post-breeding period. By doing so, they were able to demonstrate that trophic level was lowest during spring migration, slightly higher on wintering grounds, and highest in the post-breeding period, which indicates a heavy reliance on fruits as opposed to insects during migration and a reversed trend on breeding grounds. This study was also notable for its use of compound-specific amino-acid analysis to expand their ability to discriminate among dietary items, although without measurements of amino acid turnover it is unclear whether these reconstructions match the time frame assumed based on bulk-protein analysis. Nevertheless, both this and the previous

study demonstrate the benefits of considering turnover during the design phase of stable isotope diet studies.

Alternately, in some circumstances it may be possible to use turnover rates to account for incomplete equilibration by algorithmically adjusting measurements of isotopic enrichment [23,104] or by applying corrections to the discrimination factors used to estimate diet [105–107]. However, this approach is computationally intensive and used infrequently. Meanwhile, many studies do not explicitly address the assumptions about turnover that underlie stable isotope-based diet reconstructions. In many cases this omission may result from a lack of species and tissue-specific estimates of turnover and, until recently, the lack of broadly applicable tools for estimating turnover rates, such as those in [16]. Oftentimes, practical considerations also lead studies to focus on easily collected tissues like blood, hair, or feather and to reconstruct diets using only one or a small set of tissues. In spite of these limitations, consideration of turnover remains a critical part of diet reconstruction with stable isotopes, and should be carefully considered by future studies.

Pinpointing dietary shifts with isotopic clocks

When the isotopic composition of the diets for a given species is known, reliable differences in turnover between tissues, compounds, and isotopes can then be used to construct isotopic clocks that can then be used to calculate the timing of diet shifts (Figure 2A) [68,108–110]. The timing of diet shifts is, in turn, immensely valuable for reconstructing the movement and feeding behavior of individual animals. Assuming that turnover was estimated using a standard first-order kinetic equation

(see above), it is simple to rearrange the equation to use the data from a single tissue to calculate the time since the diet shift. This rearranged equation takes the form:

$$t_{est} = -\tau \times \ln \frac{(y_t - y_{\infty})}{(y_0 - y_{\infty})}$$

where t_{est} is the estimated time elapsed since the diet shift, τ is the mean retention time of the tissue or compound, y_t is the isotopic enrichment of the tissue at the time of measurement, y_0 is the enrichment at equilibrium with the initial diet, and y_{∞} is the enrichment at equilibrium with the second diet. To produce more precise estimates of the time since the diet shift, estimates from different tissues can be averaged (Figure 2C) or different equations can be derived to directly incorporate data from multiple tissues [108]. Isotopic enrichment data for multiple tissues can also be used more generally to place the timing of a dietary shift by finding the tissue with the fastest turnover that is not at equilibrium with the current diet (Figure 2B).

With the recent development of allometric equations to estimate turnover rates for novel taxa [16], as well as the increasing number of studies that have experimentally measured turnover, isotopic clocks are becoming much more widely applicable. However, they remain most applicable to species that consume resources from a range of discrete habitats or have regular shifts in their diets, particularly species that migrate between isotopically distinct habitats or exploit seasonally available resources. For example, Moore et al. [111] used a combination of carbon and sulfur isotopic clocks to estimate the minimum amount of time that juvenile pacific salmon spent in an estuary situated between their freshwater spawning grounds and marine habitats where they spend the bulk of their life. By doing so, they demonstrated that salmon do use estuaries as stopover sites along their downstream

migration and can accumulate substantial growth there, although the importance varied among species with Chinook (*Oncorhynchus tshawytscha*) and Pink Salmon (*O. gorbuscha*) remaining longest. The authors were also able to infer the existence of a unique estuarine fry life history stage in Coho Salmon (*O. kisutch*), based on the observation of a negative relationship between time spent in the estuary and body size. Finally, Moore et al. used a combination of isotopic clocks and genetic stock assessment to demonstrate population-specific differences in the use of estuaries by Sockeye Salmon (*O. nerka*), indicating within-species divergence in migratory strategies.

A similar example is a recent study by Catry et al. [112], in which they estimated the stopover lengths of a migratory shorebird, the Dunlin (*Calidris alpina*), in the Tagus estuary of Portugal. In this case they used carbon and nitrogen clocks for plasma and red blood cells to estimate time since arrival at the stopover site and linked time since arrival to stopover length with a simulation approach. Using this method, Catry et al. estimated that Dunlin spent an average of 7.5 days in the Tagus estuary during their spring migration from Mauritania to Iceland. Additionally, by combining this estimate of stopover duration with the ratio of migratory to wintering individuals in the estuary and counts of the total number of Dunlin on each day of the migratory period, the authors were able to estimate the total population of migratory individuals that used that stopover site. The resulting estimate of ~30,000 birds suggests that at least 4% of that population of Dunlin uses this one site, key information for linking the migratory ecology of this species with conservation action.

A final example of the utility of isotopic clocks is a study conducted by Boggie et al. [113], in which the authors both estimated the timing of arrival by migratory Sandhill Cranes (*Antigone canadensis*) on wintering grounds in the Middle Rio Grande valley of New Mexico and their use of anthropogenic subsidies there. Using carbon isotopic clocks for liver and muscle, the authors estimated that, on average, this population of cranes arrived on the wintering grounds in early November and subsequently relied on corn grown on state and federal lands for approximately 60% of their diet, resources that would otherwise likely be acquired by foraging on agricultural land. Results such as these are essential to guide the actions taken by state and federal agencies to most efficiently manage wildlife populations and reduce human-wildlife conflicts. More broadly, this and the previous studies demonstrate the value of isotopic clocks for estimating the timing of animal movements and diet shifts, which can then be applied to answer a wide array of ecological questions.

Inferring past and present nutritional status from turnover and isotopic signatures

Nutrition is a key aspect of animal ecology, both as a motivational force guiding the behavior of individuals and as a link between habitat and behavior, individual fitness, and corresponding population-level effects. Correspondingly, assessments of the nutritional status of individuals are an important element of a wide range of ecological studies. Several methods have been developed that use stable isotopes to assess the nutritional status of animals, each with important connections to the turnover rates of isotopes.

First, there are methods that directly use turnover rates to estimate nutritional status. As described above, catabolism and anabolism of tissue are balanced in animals at steady state, and so measurements of catabolic turnover in such animals are directly related to the supply of replacement molecules and minimum dietary requirements in the case of essential nutrients. Comparison of requirements with the actual intake of nutrients can therefore be used to assess the nutritional status of animals for whom turnover rates have been measured (Figure 3A). A related approach involves measuring the turnover of a stable isotope-labeled tracer as well as the overall balance of the tracer compound in the tissue of interest [114,115]. In this method, the disappearance of the tracer represents the rate of catabolism of the tissue containing the tracer compound, whereas the sum of the breakdown and the total balance represents the rate of tissue synthesis. Nutritional stress can then be inferred from higher rates of catabolism and lower rates of synthesis and nutritional satiety can be inferred from balanced rates of catabolism and synthesis.

Alternately, it is possible to assess nutritional status by its effect on the isotopic composition of tissues. Food deprivation, in particular, typically results in elevated $\delta^{15}\text{N}$ values in tissues, due to preferential retention of the heavier isotope during repeated de- and trans- amination of amino acids [116–121]. Fasting also seems to be related to changes in the $\delta^{13}\text{C}$ enrichment, but the observed effects have been much less consistent with elevated $\delta^{13}\text{C}$ in chicken (*Gallus gallus*) hemoglobin [122] and Bonobo (*Pan paniscus*) urine [117], but mixed results in human hair [118,121], and no effect on the muscle or liver in Ross' Geese (*Chen rossii*) [116]. Isotopic niche size is also related to nutritional status, typically expanding when

animals encounter stressful environments such as limited resource availability, and retracting in benign environments [119,120,123]. For example, Karlson, et al. [124] demonstrated that nutritional stress, parasite infestation, and toxin contamination all resulted in larger carbon-nitrogen isotope niches in the amphipod *Monoporeia affinis*, with nutritional stress producing the largest absolute change in isotopic niche space.

As with diet reconstructions, the use of isotopic signatures and niche space to assess nutritional status is facilitated by consideration of the turnover rates of sampled tissues (Figure 3B). Specifically, the estimated timing of a period of nutritional stress will depend on the turnover rate of the sampled tissue and estimates of turnover are necessary to test assumptions about timing and effectively design sampling protocols. Furthermore, studies that incorporate cross-tissue comparisons will usually be able to more accurately reconstruct timelines of nutritional state in their study taxa. However, most work linking isotopes and nutritional state, both methods directly incorporating and indirectly considering turnover, have thus far focused on humans (e.g. [115,119,120]), and so further development of these methods is likely necessary before they can be readily applied to a wide range of wild species and natural contexts.

The future of isotopic turnover

Thus far we have described the process and importance of estimating turnover rates for ecological studies that make use of stable isotope-based techniques. Below we briefly outline some of the future opportunities and challenges that isotopic turnover studies may face, including developing a more detailed mechanistic understanding of

isotopic turnover, integrating isotope clock information about resource use with direct tracking of movements, and adapting turnover-based methods in nutrition for use on wildlife.

What mechanisms drive the turnover of isotopes?

In the sections above we discussed how understanding variation in turnover rates among tissues, individuals, and species allows for more exact and effective diet reconstructions and isotopic clocks. It follows that more precise knowledge of the mechanisms that contribute to such variation can further refine these ecological methods. Developing mechanistic models of turnover will be a particularly important step towards understanding the allometry of turnover and improving predictions of turnover rates in novel taxa, thereby broadening their application to ecological studies. As we have described, there is already evidence suggesting that protein synthesis and degradation, rather than metabolic rate, are key mechanisms driving the turnover of non-lipid tissue components. Thus, while more studies are needed to verify this relationship, another important follow-up step is to integrate knowledge of the regulation of protein synthesis with turnover rates. Specific questions of interest include the 1) links between isotopic and cellular or organelle turnover, perhaps contributing to rhythmic cycles of isotopic turnover or differences among tissues, 2) the relationship between the synthesis of specific proteins and isotopic turnover and 3) the influence of dietary availability and cycling of amino acids among tissues on isotopic turnover. Moving forward, it will also be important to ask similar questions

about the turnover of other tissue components, particularly lipids, which may have different relationships with metabolism or among tissues than protein.

How can turnover be integrated with tracking technologies?

In recent years there have been tremendous advances in the number and sophistication of tracking technologies available to ecologists [125,126]. Since one of the major ecological applications of stable isotopes is for tracking the location and timing of animal movements, there is an excellent opportunity to cross-validate the results of these methods. A particularly important step is to test the precision of estimates of departure and arrival times estimated from stable isotope data with isotopic clocks. Moreover, by pairing movement histories with isotope data it may be possible to assess the effect of fasting and exercise on stable isotope signatures in a natural context. The combination of these effects with a greater mechanistic understanding of turnover could greatly refine stable isotope methods. Meanwhile, the ability to pair stable isotope data with movement data creates the possibility of more detailed analyses of resource use during large scale movements, such as diet selection at migratory stopover sites. These finer details are likely to be an important supplement to data collected from coarser tracking technologies, such as light-level geolocators and may provide new insights into the motivations for animal movements.

How can turnover inform wildlife nutrition studies?

As we discussed above, methods that link isotopic turnover to diet requirements and nutritional state or infer past nutritional stress from isotopic signatures have mostly been pursued in human contexts and will require some development before they can be reliably used to study the nutrition of wild animals. At a minimum, turnover rates of multiple tissues will need to be estimated and the isotopic signatures of nutritional stress will need to be established with greater confidence for a wider range of species. Subsequently, it will be necessary to empirically test the predictions for nutritional time series based on differences in turnover among tissues. As with ecological stable isotope methods, this process will be facilitated by a more detailed, mechanistic understanding of the drivers of turnover, which may elucidate any differences among tissues, individuals, and species. In addition to general mechanisms driving turnover, it will be important to clarify mechanisms specific to nutritional stress such as changes in routing and rates of *de novo* synthesis of tissue components. Investment in such challenges will further improve stable isotope-based assessments of nutritional state and history in ecological studies, thereby expanding our understanding of the motivations and success of animals.

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Figures

Figure 1. The process of estimating the turnover of tissues and specific compounds in animals using stable isotopes. Commonly used isotopic labels (A) include heavy isotopes of hydrogen, carbon, nitrogen, oxygen, and sulfur, which are then applied to different compounds of interest (B). The enrichment of the labeled compound(s) in animal tissues is then manipulated (C) with either a shift in diet or the administration of a dose of uniquely labeled molecules and then tissue samples (D) are collected over time following the manipulation. Tissue sampling is typically concentrated in the period immediately following the manipulation, when changes in tissue isotope enrichment are most rapid. Isotopic enrichment of tissues is then measured by mass spectrometry (E), which separates isotopes by mass and may be preceded by a gas chromatography step to distinguish between compounds of similar class. Finally, the

rate of change in isotopic enrichment is estimated (F), typically by fitting a first-order kinetic model to the data (formula shown). Models of turnover are illustrated with a comparison of the carbon turnover of neutral and polar lipids in Zebra Finch flight muscle from Carter et al. [64]. Please see the text for more details.

Figure 2.

Differences in carbon turnover among Zebra Finch tissues and their application to isotopic clocks. The ^{13}C half-lives of different tissues (A) are widely distributed over the course of a month, with 50% of liver phospholipids replaced in less than two days and 50% of leg muscle protein replaced in twenty days. Differences in turnover among tissues leads, in turn, to differences in the amount of time required for tissue isotopic enrichment to reach equilibrium with current diet (B). Duration of time required for ca. 95% of the carbon to be replaced (and hence be effectively in equilibrium with current diet) can be roughly estimated as $4.3 \cdot \text{half-life in days}$ (e.g., small intestine = $4.3 \cdot 6 \text{ days} = 26 \text{ days}$). Inset (C) depicts an isotopic clock using liver and pectoral muscle turnover rates to estimate the timing of a hypothetical

dietary shift from $\delta^{13}\text{C} = -16.3\text{‰}$ to $\delta^{13}\text{C} = -26.3\text{‰}$. For example, a measured value of $\delta^{13}\text{C} = -20.3\text{‰}$ for both liver and pectoral muscle would indicate a diet shift occurred 8-10 days ago. Data are from [64], [68], and unpublished data (Carter).

Figure 3.

Schematic illustrations of the influence of turnover on nutritional state and isotopic reconstructions of nutritional status. The balance between whole-body catabolic turnover rate (summed from individual tissue turnover rates denoted by tissue icons) and the rate of dietary intake and *de novo* synthesis can be used to assess an individual's nutritional status (A), with low intake leading to nutritional stress and high intake leading to a nutritional surplus. Differences in turnover rates among

tissues lead to differing patterns of isotopic signatures in response to diet restriction (B-D). Dotted lines in each panel are estimates of $\delta^{13}\text{C}$ of tissues if no diet restriction occurred and birds were in complete equilibrium with diet. Pictured are hypothetical differences in Zebra Finch tissue $\delta^{13}\text{C}$ following four weeks of diet restriction (B), a subsequent two weeks of *ad libitum* feeding (C), and a final week of diet restriction (D), assuming ^{13}C enrichment during diet restriction. Relative differences in $\delta^{13}\text{C}$ between tissues in the same individual(s) are indicative of diet restriction and its timing given certain assumptions about the equilibrium with diet.

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