Journal of Comparative Physiology B (2021) 191:357–370
https://doi.org/10.1007/s00360-021-01345-1

ORIGINAL PAPER

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

**Wales A. Carter[1] · Kristen J. DeMoranville[1] · Barbara J. Pierce[2] · Scott R. McWilliams[1]**

Received: 4 March 2020 / Revised: 18 December 2020 / Accepted: 18 January 2021 / Published online: 9 February 2021


## 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 elevated proportions of dietary linoleic acid (18:2*n*6) and amounts of dietary anthocyanins (a hydrophilic antioxidant class) influence the activity and protein 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 18:2*n*6-dependent relationships with soluble protein concentration. Lactate dehydrogenase (LDH) was similarly related to protein concentration although also dependent on both dietary anthocyanins and flight training. 3-Hydroxyacyl CoA Dehydrogenase (HOAD) activity increased throughout the experiment in flight muscle, whereas this relationship was dependent on dietary anthocyanins in the leg muscle. Soluble protein concentration also increased throughout the experiment in the flight muscle, but was unrelated to date in the leg muscle, instead being influenced by both dietary anthocyanins and flight training. Training also produced additive increases in CPT and leg muscle HOAD activity. FAT/CD36 expression was related to both dietary 18:2*n*6 and training and changed over the course of the experiment. These results demonstrate a notable influence of our diet manipulations and flight 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 · Enzyme activity · Citrate synthase · Carnitine palmitoyl transferase · Songbirds

## 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 support both 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 et al. 2020), and maximum speed (McKenzie and Higgs 1998). Such effects have been observed in a wide range of species, but have been particularly well-documented in songbirds (Pierce and McWilliams 2014; 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,

Communicated by P. Withers.

✉ Wales A. Carter
wales_carter@uri.edu

[1] Department of Natural Resources Science, University of Rhode Island, 1 Greenhouse Rd, Kingston, RI 02881, USA

[2] Department of Biology, Sacred Heart University, Fairfield, CT 06825, USA

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 polyunsaturated fatty acids (PUFA) upregulating their expression with the activation of peroxisome proliferator-activated receptor (PPAR) pathways (Sampath and Ntambi 2004; Marion-Letellier et al. 2016; Hamilton et al. 2018). 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 these studies did not compare diets formulated to investigate the specific effects of linoleic acid (18:2*n*6), a PUFA implicated by several studies of organism-level performance (Ayre and Hulbert 1997; McKenzie and Higgs 1998; Pierce et al. 2005; Mcwilliams et al. 2020).

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 investigated the interacting effects of dietary 18:2*n*6, 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 18:2*n*6 increases activity and expression of transporters and oxidative enzymes, (2) elevated dietary antioxidants may reduce damage to tissue components, including enzymes, and a greater proportion of functioning enzymes could therefore increase enzyme activity and expression, (3) flight training increases enzyme activity and protein 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 18:2*n*6 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. Throughout, we also consider the impact of several variables that emerged as artefacts of our experimental design on the activity and expression of lipid transporters and oxidative enzymes. Most notably, these include soluble protein content, a characteristic of tissue-level muscle condition that is often used to simply standardize enzyme activity in a sample, and date, a byproduct of being able to fly a limited number of birds at a time in the wind tunnel. To our knowledge, this is the first study to experimentally investigate the interacting effects of training, dietary anthocyanins, and dietary fatty acids on songbird enzyme activity.

## 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 2.4 m × 3.7 m × 3.1 m and two 2.4 m × 2.3 m × 3.5 m). 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. 2020): birds in two aviaries received a 32% 18:2*n*6 diet and two others received a 14% 18:2*n*6 diet. Manipulating fatty acid compositions of diets requires trading off between concentrations of multiple fatty acids. In this case we primarily traded 18:2*n*6 off with palmitic acid, 16:0, one of the fatty acids most readily synthesized by vertebrates (Klasing 1998), although there were relatively small differences between diets in other fatty acids (Table 1). However, the differences in 18:2*n*6 and 16:0 concentrations were an order of magnitude greater than any other difference between diets (Table 1) and so we have primarily assessed our results in the context of that manipulation. All of these fatty acids occur within fruits regularly consumed by migratory songbirds in eastern North America (Zurovchak 1997; Boyles 2011). On September 1, we began adding supplementary anthocyanins (elderberry powder; Artemis International, Fort Wayne, IN) to the diets of one 32% 18:2*n*6 and one 14% 18:2*n*6 aviary, producing a 2×2 factorial diet manipulation with four groups: 32% 18:2*n*6 high anthocyanin ($N = 28$), 32% 18:2*n*6 low anthocyanin ($N = 27$), 14% 18:2*n*6 high anthocyanin ($N = 27$), and 14% 18:2*n*6 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 11 h light: 13 h dark and assigned the birds to 20 single-diet cohorts of 5–6 individuals based on molt score. Beginning on September 23, and continuing every 3 days thereafter in descending order of molt score, we removed birds of the appropriate cohort from their aviary and placed them in individual 0.6 m × 0.5 m × 0.5 m cages to begin a 26-day process of flight training. Details about flight training can be found in our previous publication (Carter et al. 2020). 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 0.8 m × 1.5 m × 2 m flight aviary. Trained birds were acquainted with the AFAR wind tunnel for 20 min 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 (20 min – 180 min) in the wind tunnel and culminated in a flight on day 24 that lasted as long as birds would voluntarily fly, up to 6 h. The mean length

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

| Ingredients | % of Dry Mass | Oil Mixture (% of plant oils) | |
|---|---|---|---|
| | | Low 18:2*n*6 | High 18:2*n*6 |
| 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 | | 0.21 ± 0.01 | 0.24 ± 0.14 |
| 14:0 | | 0.91 ± 0.28 | 0.38 ± 0.04 |
| 16:0 | | 29.64 ± 0.88 | 8.65 ± 0.41 |
| 16:1*n*7 | | 0.41 ± 0.01 | 0.3 ± 0.01 |
| 18:0 | | 3.9 ± 0.14 | 2.83 ± 0.09 |
| 18:1*n*9 | | 42.76 ± 0.53 | 45.2 ± 0.57 |
| 18:2*n*6 | | 13.86 ± 0.66 | 31.4 ± 0.59 |
| 18:3*n*3 | | 2.68 ± 0.05 | 4.05 ± 0.12 |
| 20:1*n*9 | | 0.4 ± 0.02 | 0.55 ± 0.03 |
| 20:4*n*6 | | 0 ± 0 | 0 ± 0 |
| 22:6*n*3 | | 0.11 ± 0.01 | 0.15 ± 0.05 |
| 24:1 | | 0.08 ± 0.03 | 0.1 ± 0.02 |

[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 18:2*n*6 diet: canola oil and palm oil, high 18:*n*6 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. Values are ± SE and only the twelve most concentrated fatty acids are listed

of this final flight was 193 min and the maximum lasted 360 min. At the same time of day (1400–1500 h) 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 h 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 100 mg of muscle tissue in 9 volumes of homogenization buffer (20 mM $Na_2HPO_4$, 0.5 mM EDTA, 0.2% BSA, 50% glycerol, 0.1% triton x-100, 50 µg/ml Aprotinin, pH 7.4) with 3 × 10 s 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 s 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-min 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 50 mM Tris buffer (pH 8.0) with 7.5 mM carnitine, 0.15 mM DTNB (5,5′-dithio-bis-[2-nitrobenzoic acid]), 0.035 mM palmitoyl CoA, and 20 µl of diluted homogenate. CS was also assayed in 50 mM Tris buffer (pH 8.0) with 0.75 mM oxaloacetic acid, 0.15 mM DTNB, 0.15 mM acetyl CoA, and 2 µl of diluted homogenate. HOAD was assayed in 50 mM imidazole buffer (pH 7.4) with 0.2 mM NADH, 1 mM EDTA, 2 mM acetoacetyl CoA, and 20 µl of diluted homogenate. LDH was also assayed in 50 mM imidazole (pH 7.4) with 5 mM DTT (DL-dithiothretiol), 0.3 mM NADH, and 2 µl of diluted homogenate. Absorbances were read at 412 nm for CPT and CS and 340 nm for HOAD and LDH and enzyme catalytic activity per gram of wet tissue was calculated as follows:

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

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

## 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. This assay does not reflect the insoluble protein in connective tissue (e.g. collagen), making it more representative of the relative expression of soluble protein (e.g. enzymes and contractile proteins) in our whole-tissue homogenates (López et al. 1993). We primarily measured soluble protein to characterize the relative composition of muscle tissue, but also used it as a covariate in analyses of enzyme activity (see below) and to standardize assays of the expression of specific proteins. We present soluble protein concentration as mg per g of wet tissue. We 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 0.5 mg/ml with Tris–glycine buffer (pH 6.8). Briefly, we incubated 75 µl 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 µl of 5% BSA in PBS buffer (pH 7.4). Next, we incubated wells for three hours at room temperature with 75 µl of a primary antibody (mouse anti-CS, NM_004077.2, or mouse anti-FAT/CD36, AB_2538312) at a concentration of 5 µl/ml in blocking buffer, washed 3 × with 0.1% PBS triton x-100 (PBST), washed 3 × with PBS, and then incubated wells overnight at 4 °C with 75 µl of secondary antibody (rabbit anti-mouse conjugated to HRP, AB_2534831) at a concentration of 5 µl/ml in blocking buffer. Finally, the wells were again washed 3 × with 0.1% PBST and 3 × with PBS, then treated with 50 mL of chemiluminescent reagent (Pierce ECL Western Blotting Substrate, Fisher Scientific, Waltham MA USA) for 1 min, and luminescence was read 2 min 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 µg/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, and Julian date. We also included soluble protein concentration as a covariate in models of enzyme activity to test whether the broader regulation and expression of protein influenced activity, and whether that influence interacted with the effects of diet and training. 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 the 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. 2020 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 fatty acids, dietary anthocyanins, training, soluble protein concentration, and date, and were also dependent on tissue type (statistical results summarized in Table 2). In pectoral muscle both CPT and CS activity were positively related to soluble protein concentration when birds were fed the 14% 18:2*n*6 diet, but negatively related when fed the 32% 18:2*n*6 diet (table S-1; CPT: Fig. 1a, $T_{87} = -2.286$, $P = 0.0247$; CS: Fig. 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% 18:2*n*6 diet, but unrelated when fed the 14% 18:2*n*6 diet (table S-2; CPT: Fig. 1b, $T_{83} = 2.027$, $P = 0.0458$; CS: Fig. 1d, $T_{83} = 1.944$, $P = 0.0553$) and negatively related to wing chord regardless of diet (table S-2; 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 S-1; $T_{86} = 2.201$, $P = 0.0304$) and flight training produced an additive increase in the activity of CPT in both pectoral (table S-1; Fig. 2A, $T_{87} = -3.149$, $P = 0.0023$) and leg muscle (table S-2; Fig. 2B, $T_{83} = -2.092$, $P = 0.0395$). LDH activity was also related to soluble protein concentration in both pectoral and leg muscle (Fig. 1e, f), but these relationships depended on, respectively, the interaction between dietary fatty acids and dietary anthocyanins (table S-1; $T_{84} = 2.391$, $P = 0.0190$) and the interaction between dietary fatty acids and flight training (table S-2; $T_{81} = 3.695$, $P = 0.0004$). HOAD activity in pectoral muscle increased over the course of the experiment (table S-1, Fig. 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 S-2, Fig. 3b; $T_{84} = 3.073$, $P = 0.0029$). Leg muscle HOAD activity increased additively with flight training (table S-2, Fig. 2c; $T_{84} = -2.075$, $P = 0.0411$). Soluble protein concentration in pectoral muscle was also positively related to date (table S-1, Fig. 3c; $T_{89} = 3.300$, $P = 0.0014$), but decreased with training (table S-2; $T_{86} = 2.516$, $P = 0.0137$) and the supplementation of dietary anthocyanins (table S-2; $T_{86} = 2.163$, $P = 0.0333$) in leg muscle (Fig. 3d). Variation in soluble protein concentration, a relative measure of composition, needs to be traded off with variation in other tissue components, such as lipids or insoluble protein. 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 S-3, Fig. 4a; $T_{16} = -2.561$, $P = 0.0209$). However, FAT/CD36 expression decreased over the course of the experiment in all experimental groups except untrained birds who were fed the 14% 18:2*n*6 diet (table S-3, Fig. 4b; $T_{12} = -2.327$, $P = 0.0383$).

### Correlations among enzyme activities and expression

In pectoral muscle (Table 3), 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 4) 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 18:2*n*6, hydrophilic dietary antioxidants, and training on the activity and expression of several key

**Table 2** Summary of the significant effects of experimental treatments and incidental variables on measures of enzyme activity, soluble protein, and enzyme expression in the flight and leg muscle of European Starlings

| Tissue | | | CPT Activity | CS Activity | HOAD Activity | LDH Activity | Soluble Protein | CS Expression | FAT/CD36 Expression |
|---|---|---|---|---|---|---|---|---|---|
| Flight muscle | Experimental Treatments | Dietary 18:2*n*6 | XX[a] | XX[a] | | XXX[c] | | | XXX[e] |
| | | Dietary anthocyanins | | | | XXX[c] | | | |
| | | Flight training | + | | | | | | XXX[e] |
| | Incidental Variables | Soluble protein | XX[a] | XX[a] | + | XXX[c] | | | |
| | | Julian date | | | | | + | | XXX[e] |
| | | Body mass | | + | | | | − | |
| Leg muscle | Experimental Treatments | Dietary 18:2*n*6 | XX[a] | XX[a] | | XXX[d] | | NA | NA |
| | | Dietary anthocyanins | | | XX[b] | | − | NA | NA |
| | | Flight training | + | | + | XXX[d] | − | NA | NA |
| | Incidental Variables | Soluble protein | XX[a] | XX[a] | | XXX[d] | | NA | NA |
| | | Julian date | | | XX[b] | | | NA | NA |
| | | Wing chord | − | − | | | | NA | NA |

Symbols indicate significantly ($p < 0.05$) positive additive (+), negative additive (−), or interactive relationships between treatments (32% vs. 14% 18:2*n*6, high vs. low anthocyanins, flight training vs. control), incidental variables, and dependent variables. Two-way interactions are denoted with XX and three-way interactions are denoted with XXX. Blank cells in the table are not significant effects ($p > 0.05$) and 'NA' indicates that dependent variables were not measured for that tissue. For the specific effects of tests with interactions, please see Figs. 1 and 4

[a]2-way interaction between dietary 18:2*n*6 and soluble protein for each tissue; [b]2-way interaction between dietary anthocyanins and Julian date; [c]3-way interaction between dietary 18:2*n*6, dietary anthocyanins, soluble protein; [d]3-way interaction between dietary 18:2*n*6, flight training, soluble protein; [e]3-way interaction between dietary 18:2*n*6, flight training, Julian date

lipid transporters and oxidative enzymes in a songbird. In general, we seldom found simple, direct effects of any of the three treatments (see Table 2 for summary) and instead found that the main effects of the treatments were revealed usually when considering the soluble protein concentration of the tissue (Table 2). For example, we found that the influence of dietary fatty acids was usually dependent on the soluble protein concentration of the muscle tissue (soluble protein × dietary 18:2*n*6 interaction, Table 2, Fig. 1), 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 (no dietary 18:2*n*6 × dietary anthocyanin interaction). We did, however, find striking differences in the activity of oxidative enzymes between flight and leg muscle (Fig. 1; Table 2), which further emphasizes the importance of tissue-specific regulation for patterns of enzyme activity and the context-dependence of any dietary influence on muscle metabolism. The mechanisms implicated by these results help to shed light on previous studies that found links between dietary 18:2*n*6 and organism-level performance. We expand on these conclusions throughout the following discussion.

## Dietary 18:2*n*6 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 18:2*n*6, has not been the focus of prior diet manipulations. Nevertheless, in the studies on songbirds where 18:2*n*6 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

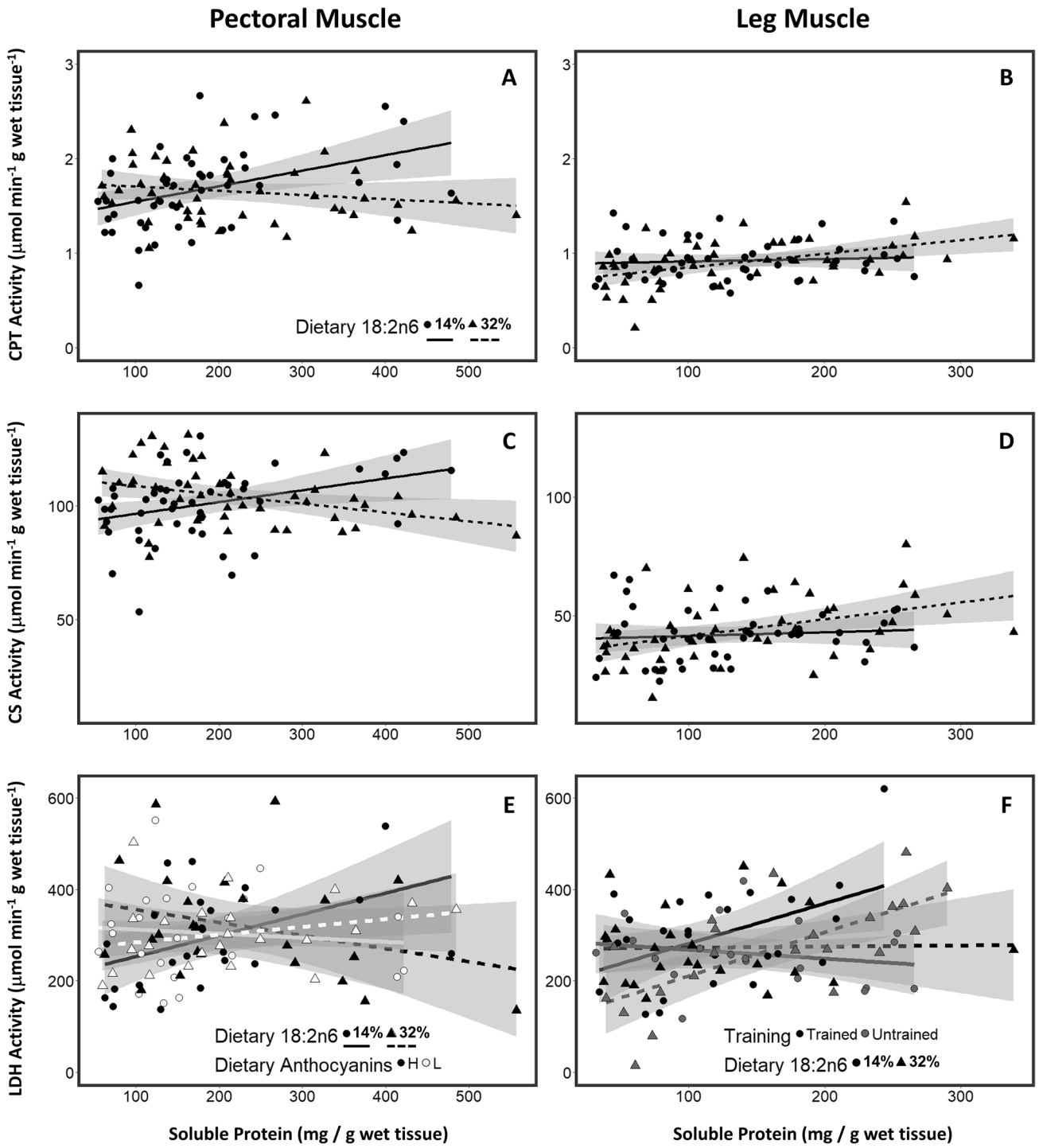


**Fig. 1** The relationships between CPT activity (**a**, **b**), CS activity (**c**, **d**), LDH activity (**e**, **f**) and soluble protein concentration in the pectoral and leg muscle of European Starlings. Birds fed the 32% 18:2*n*6 diet (triangles, dashed lines) differed from those fed the 14% 18:2*n*6 diet (circles, solid lines). LDH activity was also dependent on anthocyanin supplementation in the pectoral muscle (E, light grey = unsupplemented) and flight training in the leg muscle (F, dark grey = untrained)

differences between our 32% 18:2*n*6 and 14% 18:2*n*6 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 18:2*n*6 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

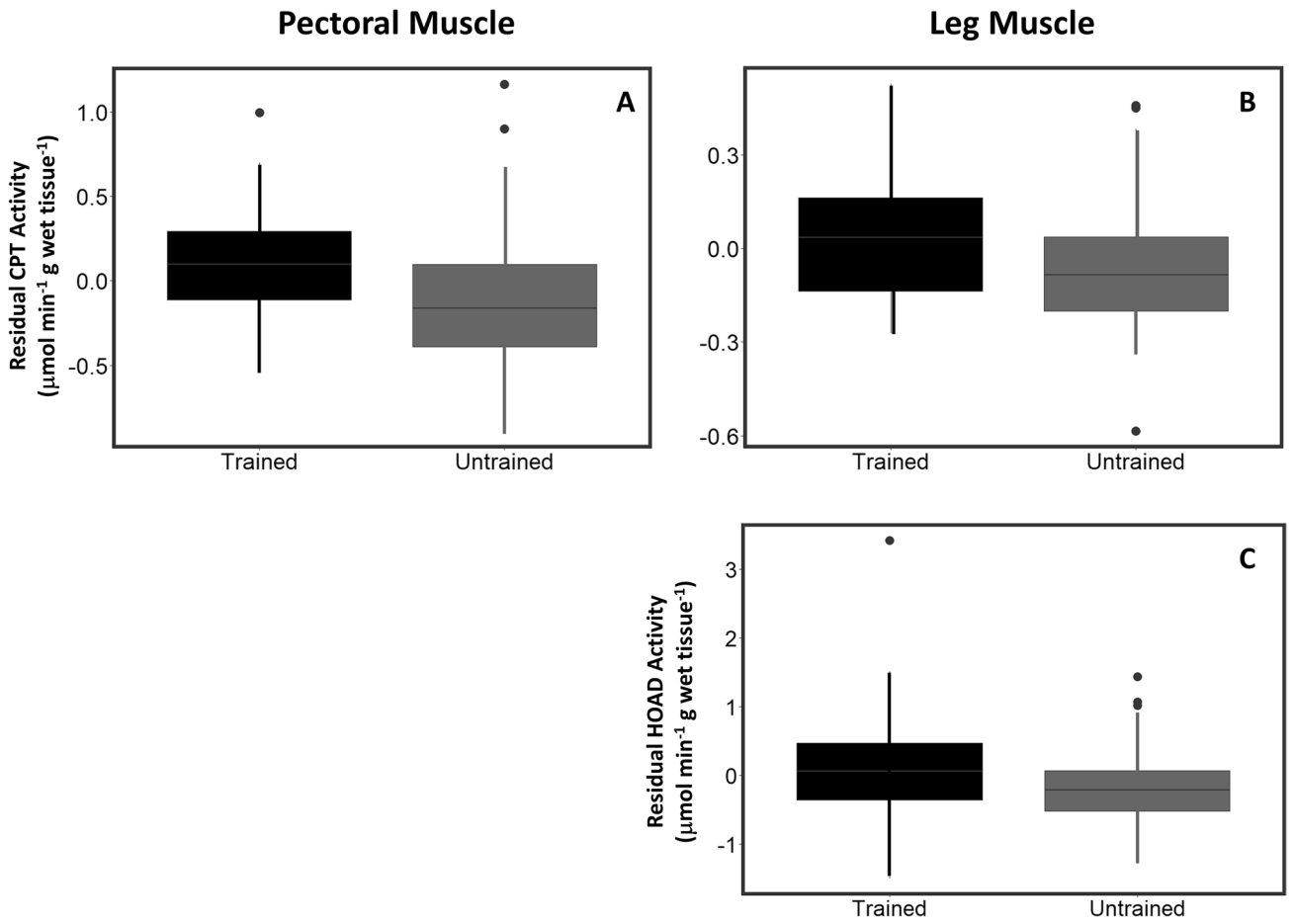


**Fig. 2** Flight training produced additive increases in the activity of CPT (**a**, **b**) in flight and leg muscle and HOAD (**c**) in the leg muscle of European Starlings. Black points indicate outliers

than simple additive differences, diet groups exhibited similar rates of CPT, CS, and LDH activity at low-to-average concentrations of soluble protein and diverged at high soluble protein concentrations in muscle tissue. These results suggest that (1) dietary 18:2*n*6 is involved in regulating the expression of CPT, CS, and LDH, and (2) that this regulation of enzyme activity occurs within the broader context of muscle condition (e.g. soluble protein content), which varies depending on the regulation of protein synthesis and regulation. 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 upregulation of muscle growth or changes in muscle composition, such as those associated with migration (Evans et al. 1992; Price et al. 2011). In any case, these results and those of other recent studies demonstrate that the expectation described in the introduction that elevated dietary 18:2*n*6 produce additive increases in the activity and expression of oxidative enzymes is far too simplistic to accurately reflect the influence of this fatty acid on muscle function and that future studies should take the broader context of muscle condition into account more explicitly.

Our diet manipulation also influenced CPT and CS activity similarly, with flight muscle in the 14% 18:2*n*6 group showing a positive relationship between enzyme activity and soluble protein and birds in the 32% 18:2*n*6 group showing a negative or null relationship between enzyme activity and soluble protein. This pattern suggests that, at high soluble protein concentrations, birds fed the 14% 18:2*n*6 diet were more oriented towards fat catabolism and capable of higher metabolic rates than birds fed the 32% 18:2*n*6 diet. Such differences in metabolic orientation run counter to the expected influence of 18:2*n*6, 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%

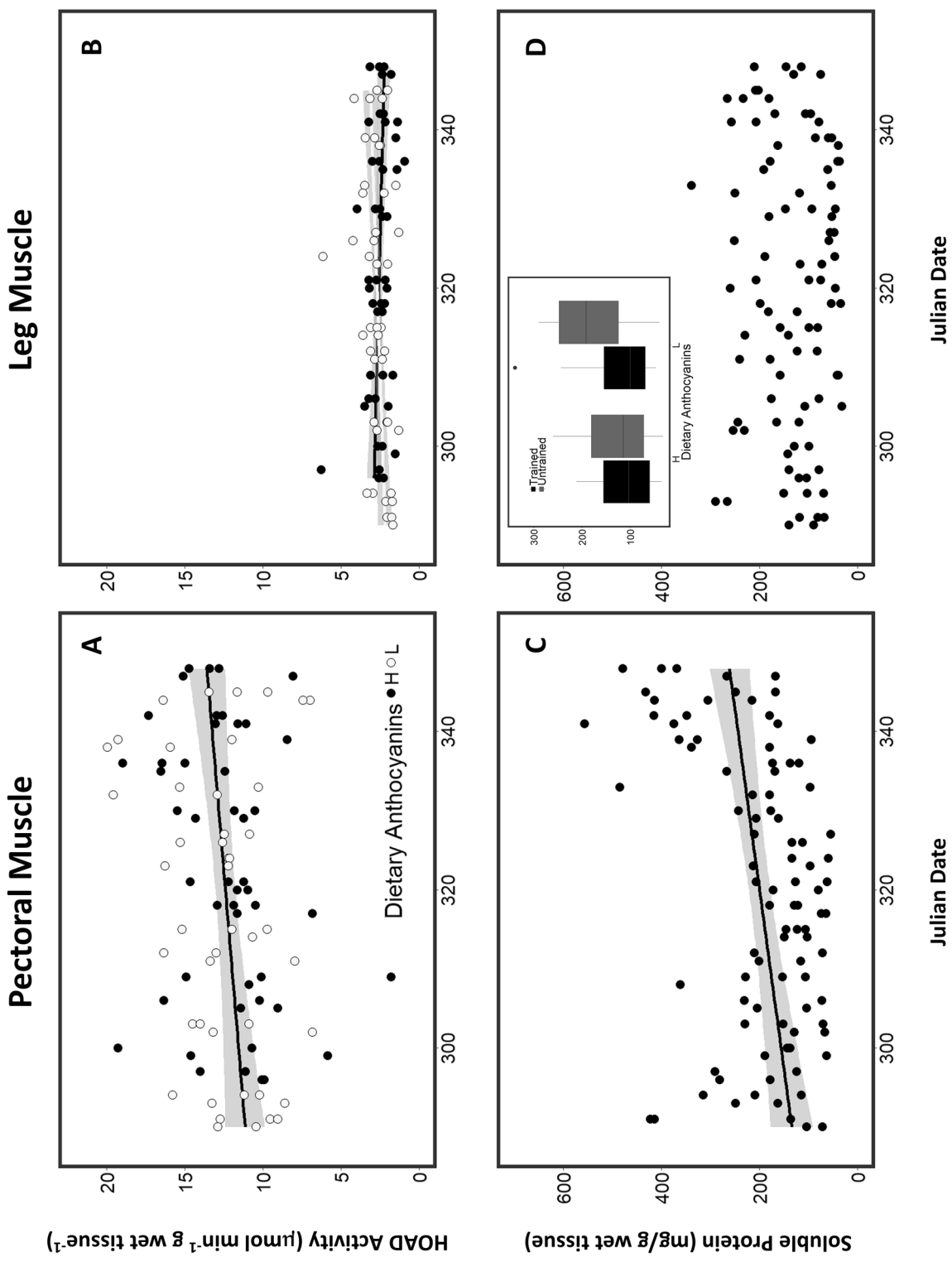


**Fig. 3** The relationships between HOAD activity (**a**, **b**), soluble protein concentration (**c**, **d**), and Julian date in pectoral and leg muscle of European Starlings. Both HOAD activity and soluble protein concentration increased over time in the pectoral muscle, whereas in the leg muscle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

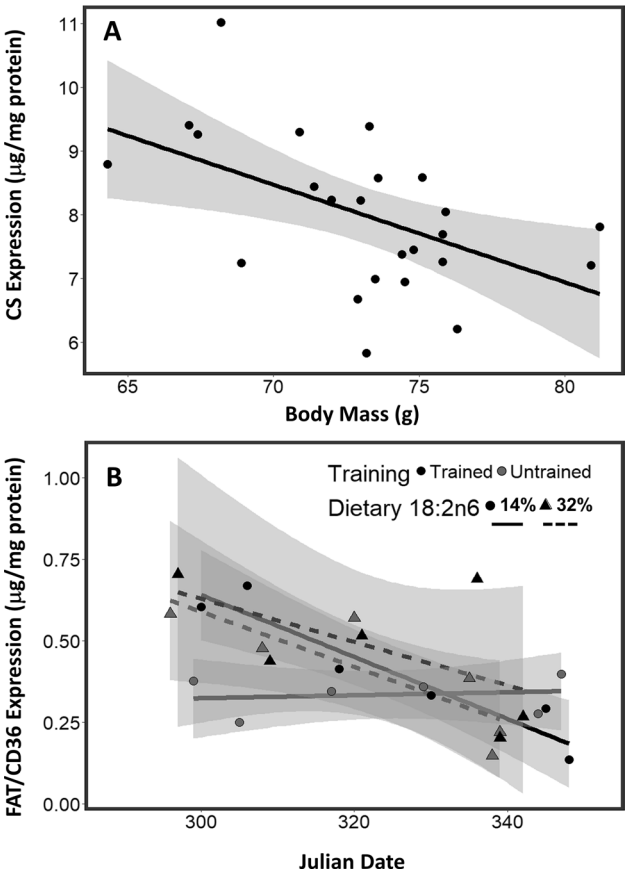


**Fig. 4** The expression of CS (**a**) and FAT/CD36 (**b**) in the pectoral muscle of European Starlings. CS concentration was negatively related to body mass, whereas FAT/CD36 exhibited dietary 18:2*n*6- (32% = dashed lines, 14% = solid lines) and flight training- (dark grey = untrained) specific changes over time

18:2*n*6 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 18:2*n*6 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% 18:2*n*6 group regardless of whether the birds were trained, suggesting that 18:2*n*6 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 14 and 32% 18:2*n*6 or trained and untrained groups for any enzyme measured (hypothesis 4), 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 (hypothesis 2; 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

**Table 3** Correlation coefficients for relationships among enzyme activities and expression in the pectoral muscle of European Starlings

| | 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 |

Asterisks denote significance, with * indicating $p < 0.05$ and ** indicating $p < 0.01$

**Table 4** Correlation coefficients for relationships among enzyme activities in the leg muscle of European Starlings

| | 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 |

Asterisks denote significance, with * indicating $p<0.05$ and ** indicating $p<0.01$

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 18:2*n*6 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. The influence of flight-based training on leg muscle is somewhat unexpected, but there are several possible explanations. First, the flight training regimen may have involved the leg muscles more than expected through changing the amount of time perching and holding the legs close to the body for flight. Second, flight training could conceivably lead to reduced or altered behavioral patterns outside of the wind tunnel that changed the use of the legs. Finally, changes to the regulation of flight muscle condition could have resulted in systemic changes that also affected the regulation of leg muscle condition. However, it is currently unclear which of these explanations is best supported.

As predicted, we also found substantial differences in the activities of CPT, CS, and HOAD between flight and leg muscle (Figs. 1, 3). These findings are consistent with the central role of these enzymes in aerobic metabolism, and the known differences in aerobic workload between flight muscle and leg muscle. However, we found that flight and leg muscle also differed substantially in their responses to dietary 18:2*n*6, dietary anthocyanins, and flight training. In particular, leg muscle was more likely to be influenced by dietary anthocyanins (two out of three enzymes across both tissues with significant effects) and training (four out of six enzymes across both tissues with significant effects) than flight muscle. These differences might suggest that the regulation of enzymes in flight muscle to higher levels of activity may outweigh most effects of dietary anthocyanins and training, whereas the lower initial activity of enzymes in leg muscle leaves such effects more visible. Flight and leg muscle also differed in how the combination of soluble protein and dietary 18:2*n*6 influenced the activity of CPT and CS. Most notably, the high 18:2*n*6 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 18:2*n*6 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 more closely investigate the regulation of soluble protein content in different tissues and in animals fed different diets. Moreover, several individuals exhibited surprisingly high concentrations in the range of 400 mg/g, which contributed to a substantial amount of variation in soluble protein content and changing concentrations over time (see below). These values are substantially higher than mean protein concentrations observed in turkeys ($210\pm10$ mg/g; Patterson et al. 2017), king penguins ($262\pm14$ mg/g; Cherel et al. 1993), and horned larks ($257\pm29$ mg/g; Swain 1992), but the overall variation in those estimates was comparable to the variation in our study, where the mean and standard error in pectoral muscle soluble protein were $202\pm12$ mg/g. Nevertheless, these findings raise questions about the normal range of protein content in songbird muscle tissue and a deeper understanding of the phenomenon of soluble protein concentration more generally is likely to be an important foundation for any future work in this field.

### Changes in enzyme activity and expression over time

We found several unexpected 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 the 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 18:2*n*6 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. 2020), 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 prepare songbirds for migration. To the extent that training can be used to prepare for migration, it appears to involve primarily changes outside of the muscle (e.g. fuel supply to the muscle) or changes to muscle size rather than changes to the function of the muscle (e.g. capacity for fatty acid catabolism), with the possible exception of fatty acid transport into the mitochondrion. 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, which have been associated with 50–100% increases in songbird enzyme activity (McFarlan et al. 2009; Zhang et al. 2015b). 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.

**Supplementary Information** The online version contains supplementary material available at https://doi.org/10.1007/s00360-021-01345-1.

**Acknowledgements** We would like to thank Morag Dick for providing us with the enzyme activity protocols used in this study, as well as valuable advice on how to successfully run them. We would also like to thank Chris Guglielmo, Andrew Gould, Mikaela Rebuli, and all of the staff at AFAR for providing us unrestricted access to the AFAR facility, for helping us think critically about the experiment, and for their support throughout. We are especially thankful for the efforts of Luke Douglas and Keara Bohannon, our undergraduate research assistants, who made the project both possible and much more enjoyable. We thank Artemis International for their kind donation of the elderberry powder used in this study. Finally, we thank Todd at Hyland Dairy Farms Ltd. for graciously allowing us to catch and remove the starlings used in this experiment on his property.

**Author contributions** SRM and BJP conceived of and designed the experiment. WAC and KJD refined the methodology and conducted the experiment. WAC analyzed the data and primarily wrote the manuscript; all other authors provided editorial advice to the manuscript.

**Funding** This research was funded by Grant Number IOS-1354187 from the National Science Foundation.

### Compliance with ethical standards

**Conflict of interest** The authors declare that they have no conflicts of interest.

## References

Alan RR, McWilliams SR (2013) Oxidative stress, circulating antioxidants, and dietary preferences in songbirds. Comp Biochem Physiol B Biochem Mol Biol 164:185–193. https://doi.org/10.1016/j.cbpb.2012.12.005

Amitai O, Bauchinger U, McCue MD, Pinshow B (2009) The effects of dietary ($n-3$) and ($n-6$) oils on basal metabolic rate in Zebra finches. In: Integrative and Comparative Biology (ICB). pp e191–e332

Anttila K, Jäntti M, Mänttäri S (2010) Effects of training on lipid metabolism in swimming muscles of sea trout (*Salmo trutta*). J Comp Physiol B Biochem Syst Environ Physiol 180:707–714. https://doi.org/10.1007/s00360-010-0446-1

Ayre KJ, Hulbert AJ (1997) Dietary fatty acid profile affects endurance in rats. Lipids 32:1265–1270. https://doi.org/10.1007/s11745-006-0162-5

Beaulieu M, Schaefer HM (2013) Rethinking the role of dietary antioxidants through the lens of self-medication. Anim Behav 86:17–24. https://doi.org/10.1016/j.anbehav.2013.05.022

Bolser JA, Alan RR, Smith AD et al (2013) Birds select fruits with more anthocyanins and phenolic compounds during autumn migration. Wilson J Ornithol 125:97–108

Boyles M (2011) Seasonal diet preferences for fatty acids differ between species of migratory passerine, are affected by antioxidant level and relate to the fatty acid composition of wild fruits. University of Rhode Island, Kingston

Cadee N, Piersmal T, Daani S (1996) Endogenous circannual rhythmicity in a non-passerine migrant, the know *Calidris canutus*. Ardea 84:75–84

Carroll CC, Carrithers JA, Trappe TA (2004) Contractile protein concentrations in human single muscle fibers. J Muscle Res Cell Motil 25:55–59. https://doi.org/10.1023/B:JURE.0000021362.55389.6b

Carter WA, Demoranville KJ, Pierce BJ, Mcwilliams SR (2020) The effects of dietary linoleic acid and hydrophilic antioxidants on basal, peak, and sustained metabolism in flight-trained European starlings. Ecol Evol 10:1552–1566. https://doi.org/10.1002/ece3.6010

Catoni C, Schaefer HM, Peters A (2008) Fruit for health: the effect of flavonoids on humoral immune response and food selection in a frugivorous bird. Funct Ecol 22:649–654. https://doi.org/10.1111/j.1365-2435.2007.0

Chepelev NL, Bennitz JD, Wright JS et al (2009) Oxidative modification of citrate synthase by peroxyl radicals and protection with novel antioxidants. J Enzyme Inhib Med Chem 24:1319–1331. https://doi.org/10.3109/14756360902852586

Cherel Y, Charrassin J, Handrich Y (1993) Comparison of body reserve buildup in prefasting chicks and adults of king penguins (*Aptenodytes patagonicus*). Physiol Zool 66:750–770

Collin A, Swennen Q, Skiba-Cassy S et al (2009) Regulation of fatty acid oxidation in chicken (*Gallus gallus*): interactions between genotype and diet composition. Comp Biochem Physiol 153:171–177. https://doi.org/10.1016/j.cbpb.2009.02.012

Cooper-Mullin C, McWilliams SR (2016) The role of the antioxidant system during intense endurance exercise: lessons from migrating birds. J Exp Biol 219:3684–3695. https://doi.org/10.1242/jeb.123992

Dick MF, Guglielmo CG (2019) Dietary polyunsaturated fatty acids influence flight muscle oxidative capacity but not endurance flight performance in a migratory songbird. Am J Physiol Regul Integr Comp Physiol 316:R362–R375

Evans PR, Davidson NC, Uttley JD, Evans RD (1992) Premigratory hypertrophy of flight muscles : an ultrastructural study. Ornis Scand 23:238–243

Fukasawa M, Atsuzawa K, Mizutani K et al (2010) Immunohistochemical localization of mitochondrial fatty acid β-oxidation enzymes in rat testis. J Histochem Cytochem 58:195–206. https://doi.org/10.1369/jhc.2009.954693

Gallagher SR (2008) Protein blotting: immunoblotting. Curr Protoc Essent Lab Tech. https://doi.org/10.1002/9780470089941.et0803s00

Ginn HB, Melville DS (1983) Moult in birds. British Trust for Ornithology, Thetford

Giroud S, Frare C, Strijkstra A et al (2013) Membrane phospholipid fatty acid composition regulates cardiac SERCA activity in a hibernator, the syrian hamster (*Mesocricetus auratus*). PLoS ONE. https://doi.org/10.1371/journal.pone.0063111

Goodman CA, Kotecki JA, Jacobs BL, Hornberger TA (2012) Muscle fiber type-dependent differences in the regulation of protein synthesis. PLoS ONE. https://doi.org/10.1371/journal.pone.0037890

Goslow GE Jr, Wilson D, Poore SO (2003) Neuromuscular correlates to the evolution of flapping flight in birds. Brain Behav Evol 55:85–99. https://doi.org/10.1159/000006644

Guglielmo CG (2010) Move that fatty acid: fuel selection and transport in migratory birds and bats. Integr Comp Biol 50:336–345. https://doi.org/10.1093/icb/icq097

Guglielmo CG (2018) Obese super athletes: fat-fueled migration in birds and bats. J Exp Biol 221:jeb165753. https://doi.org/10.1242/jeb.165753

Guglielmo CG, Haunerland NH, Hochachka PW, Williams TD (2002) Seasonal dynamics of flight muscle fatty acid binding protein and catabolic enzymes in a migratory shorebird. Am J Physiol Regul Integr Comp Physiol 282:R1405–R1413. https://doi.org/10.1152/ajpregu.00267.2001

Halliwell B, Gutteridge J (2007) Free radicals in biology and medicine, 4th edn. Oxford University Press, Oxford

Hamilton A, Ly J, Robinson JR, Corder KR, DeMoranville KJ, Schaeffer PJ, Huss JM (2018) Conserved transcriptional activity and ligand responsiveness of avian PPARs: potential role in regulating lipid metabolism in mirgratory birds. Gen Comp Endocrinol 268:110–120. https://doi.org/10.1016/j.ygcen.2018.08.009

Hashimoto M, Inoue T, Katakura M et al (2016) Differential effects of docoosahexaenoic and arachidonic acid on fatty acid composition and myosin heavy chain-related genes of slow- and fast-twitch skeletal muscle tissues. Mol Cell Biochem 415:169–181. https://doi.org/10.1007/s11010-016-2689-y

Helge JW, Ayre K, Chaunchaiyakul S et al (1998) Endurance in high-fat-fed rats: effects of carbohydrate content and fatty acid profile. J Appl Physiol 85:1342–1348

Helge JW, Wu BJ, Willer M et al (2001) Training affects muscle phopholipid fatty acid composition in humans. J Appl Physiol 90:670–677

Hulbert AJ (2010) Metabolism and longevity: Is there a role for membrane fatty acids? Integr Comp Biol 50:808–817. https://doi.org/10.1093/icb/icq007

Jenni-Eiermann S, Jenni L, Smith S, Costantini D (2014) Oxidative stress in endurance flight: an unconsidered factor in bird migration. PLoS ONE 9:e97650. https://doi.org/10.1371/journal.pone.0097650

Kim E, Park H, Cha Y-S (2004) Exercise training and supplementation with carnitine and antioxidants increases carnitine stores, triglyceride utilization and endurance in exercising rats. J Nutr Sci Vitaminol 50:335–343

Klasing KC (1998) Comparative avian nutrition. CAB International, Wallingford

Larcombe SD, Tregaskes CA, Coffey JS et al (2008) The effects of short-term antioxidant supplementation on oxidative stress and flight performance in adult budgerigars *Melopsittacus undulatus*. J Exp Biol 211:2859–2864. https://doi.org/10.1242/jeb.017970

Lihoreau M, Buhl J, Charleston MA et al (2015) Nutritional ecology beyond the individual: a conceptual framework for integrating nutrition and social interactions. Ecol Lett 18:273–286. https://doi.org/10.1111/ele.12406

López JM, Imperial S, Valderrama R, Navarro S (1993) An improved bradford protein assay for collagen proteins. Clin Chim Acta 220:91–100. https://doi.org/10.1016/0009-8981(93)90009-S

Lundgren BO, Kiessling K-H (1986) Catabolic enzyme activities in the pectoralis muscle of premigratory and migratory juvenile Reed Warblers (Herm.). Oecologia 68:529–532

Lundgren BO, Kiessling KH (1988) Comparative aspects of fibre types, areas, and capillary supply in the pectoralis muscle of some passerine birds with differing migratory behaviour. J Comp Physiol B 158:165–173. https://doi.org/10.1007/BF01075830

Maillet D, Weber J-M (2007) Relationship between n-3 PUFA content and energy metabolism in the flight muscles of a migrating shorebird: evidence for natural doping. J Exp Biol 210:413–420. https://doi.org/10.1242/jeb.02660

Marion-Letellier R, Savoye G, Ghosh S (2016) Fatty acids, eicosanoids and PPAR gamma. Eur J Pharmacol 785:44–49. https://doi.org/10.1016/j.ejphar.2015.11.004

Martinez del Rio C, McWilliams SR (2016) How essential fats affect bird performance and link aquatic ecosystems and terrestrial consumers. Proc Natl Acad Sci 113:11988–11990. https://doi.org/10.1073/pnas.1614106113

Mataix J, Quiles JL, Huertas JR et al (1998) Tissue specific interactions of exercise, dietary fatty acids, and vitamin e in lipid peroxidation. Free Radic Biol Med 24:511–521. https://doi.org/10.1016/S0891-5849(97)00288-8

McClelland GB (2004) Fat to the fire: the regulation of lipid oxidation with exercise and environmental stress. Comp Biochem Physiol B Biochem Mol Biol 139:443–460. https://doi.org/10.1016/j.cbpc.2004.07.003

McDonald P, Edwards RA, Greenhalgh JFD et al (2011) Animial nutrition, 7th edn. Pearson, Harlow

McFarlan JT, Bonen A, Guglielmo CG (2009) Seasonal upregulation of fatty acid transporters in flight muscles of migratory white-throated sparrows (*Zonotrichia albicollis*). J Exp Biol 212:2934–2940. https://doi.org/10.1242/jeb.031682

McKenzie D, Higgs D (1998) Dietary fatty acid composition influences swimming performance in Atlantic salmon (Salmo salar) in seawater. Fish Physiol Biochem 19:111–122

Mcwilliams S, Pierce B, Wittenzellner A et al (2020) The energy savings-oxidative cost trade- off for migratory birds during endurance flight. Elife 8:1–18

Mowry A, Donoviel Z, AN K, Hood W (2017) Mitochondrial function and bioenergetic tradeoffs during lactation in the House Mouse (*Mus musculus*). Ecol Evol. https://doi.org/10.1002/ece3.2817

Murphy ME, King JR (1982) Semi-synthetic diets as a tool for nutritional ecology. Auk 99 (1):165–167

Mustonen A, Käkelä R, Asikainen J, Nieminen P (2009) Selective fatty acid mobilization from adipose tissues of the pheasant (*Phasianus colchicus mongolicus*) during food deprivation. Physiol Biochem Zool 82:531–540. https://doi.org/10.1086/605393

Nagahuedi S, Popesku JT, Trudeau VL, Weber J-M (2009) Mimicking the natural doping of migrant sandpipers in sedentary quails: effects of dietary $n-3$ fatty acids on muscle membranes and PPAR expression. J Exp Biol 212:1106–1114. https://doi.org/10.1242/jeb.027888

Patterson BA, Matarneh SK, Stufft KM et al (2017) Pectoralis major muscle of Turkey displays divergent function as correlated with meat quality. Poult Sci 96:1492–1503. https://doi.org/10.3382/ps/pew410

Pierce BJ, McWilliams SR (2014) The fat of the matter: How dietary fatty acids can affect exercise performance. Integr Comp Biol 54:903–912. https://doi.org/10.1093/icb/icu098

Pierce BJ, McWilliams SR, O'Connor TP et al (2005) Effect of dietary fatty acid composition on depot fat and exercise performance in a migrating songbird, the red-eyed vireo. J Exp Biol 208:1277–1285. https://doi.org/10.1242/jeb.01493

Price ER (2010) Dietary lipid composition and avian migratory flight performance: development of a theoretical framework for avian fat storage. Comp Biochem Physiol A Mol Integr Physiol 157:297–309. https://doi.org/10.1016/j.cbpa.2010.05.019

Price ER, Guglielmo CG (2009) The effect of muscle phospholipid fatty acid composition on exercise performance: a direct test in the migratory white-throated sparrow (*Zonotrichia albicollis*). Am J Physiol Regul Integr Comp Physiol 297:R775–R782. https://doi.org/10.1152/ajpregu.00150.2009

Price ER, Krokfors A, Guglielmo CG (2008) Selective mobilization of fatty acids from adipose tissue in migratory birds. J Exp Biol 211:29–34. https://doi.org/10.1242/jeb.009340

Price ER, McFarlan JT, Guglielmo CG (2010) Preparing for migration? The effects of photoperiod and exercise on muscle oxidative enzymes, lipid transporters, and phospholipids in white-crowned sparrows. Physiol Biochem Zool 83:252–262. https://doi.org/10.1086/605394

Price ER, Bauchinger U, Zajac DM et al (2011) Migration- and exercise-induced changes to flight muscle size in migratory birds and association with IGF1 and myostatin mRNA expression. J Exp Biol 214:2823–2831. https://doi.org/10.1242/jeb.057620

Raclot T (2003) Selective mobilization of fatty acids from adipose tissue triacylglycerols. Prog Lipid Res 42:257–288. https://doi.org/10.1016/S0163-7827(02)00066-8

Rani S, Kumar V (2013) General and comparative endocrinology avian circannual systems: persistence and sex differences. Gen Comp Endocrinol 190:61–67. https://doi.org/10.1016/j.ygcen.2013.04.013

Sampath H, Ntambi JM (2004) Polyunsaturated fatty acid regulation of gene expression. Nutr Rev 62:333–339. https://doi.org/10.1301/nr.2004.sept.333

Siu PM, Donley DA, Bryner RW, Alway SE (2003) Citrate synthase expression and enzyme activity after endurance training in cardiac and skeletal muscles. J Appl Physiol 94:555–560

Skrip MM, McWilliams SR (2016) Oxidative balance in birds: an atoms-to-organisms-to-ecology primer for ornithologists. J Field Ornithol 87:1–20. https://doi.org/10.1111/jofo.12135

Strobel NA, Peake JM, Matsumoto AYA et al (2011) Antioxidant supplementation reduces skeletal muscle mitochondrial biogenesis. Med Sci Sports Exerc 43:1017–1024. https://doi.org/10.1249/MSS.0b013e318203afa3

Swain SD (1992) Flight muscle catabolism during overnight fasting in a passerine bird, *Eremophila alpestris*. J Comp Physiol B 162:383–392. https://doi.org/10.1007/BF00260767

Turner N, Haga KL, Else PL, Hulbert AJ (2006) Scaling of $Na^+$, $K^+$-ATPase molecular activity and membrane fatty acid composition in mammalian and avian hearts. Physiol Biochem Zool 79:522–533. https://doi.org/10.1086/502815 (**PBZ040164[pii]r**)

Twining CW, Brenna JT, Hairston NG, Flecker AS (2016a) Highly unsaturated fatty acids in nature: What we know and what we need to learn. Oikos 125:749–760. https://doi.org/10.1111/oik.02910

Twining CW, Brenna JT, Lawrence P et al (2016b) Omega-3 long-chain polyunsaturated fatty acids support aerial insectivore performance more than food quantity. Proc Natl Acad Sci 113:10920–10925. https://doi.org/10.1073/pnas.1603998113

Weber J-M (2011) Metabolic fuels: regulating fluxes to select mix. J Exp Biol 214:286–294. https://doi.org/10.1242/jeb.047050

Zajac DM, Cerasale DJ, Landman S, Guglielmo CG (2011) Behavioral and physiological effects of photoperiod-induced migratory state and leptin on *Zonotrichia albicollis*: II. Effects on fatty acid metabolism. Gen Comp Endocrinol 174:269–275. https://doi.org/10.1016/j.ygcen.2011.08.024

Zhang Y, Carter T, Eyster K, Swanson DL (2015a) Acute cold and exercise training up-regulate similar aspects of fatty acid transport and catabolism in house sparrows (*Passer domesticus*). J Exp Biol 218:3885–3893. https://doi.org/10.1242/jeb.126128

Zhang Y, King MO, Harmon E, Swanson DL (2015b) Summer-to-winter phenotypic flexibility of fatty acid transport and catabolism in skeletal muscle and heart of small birds. Physiol Biochem Zool 88:535–549. https://doi.org/10.1086/682154

Zurovchak JG (1997) Nutritional role of high-lipid fruits in the diet of migrant thrushes. Rutgers University, New Brunswick