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RESEARCH ARTICLE

The effects of training, acute exercise and dietary fatty acid composition on muscle lipid oxidative capacity in European starlings

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ABSTRACT

Migratory birds undergo seasonal changes to muscle biochemistry. Nonetheless, it is unclear to what extent these changes are attributable to the exercise of flight itself versus endogenous changes. Using starlings (*Sturnus vulgaris*) flying in a wind tunnel, we tested the effects of exercise training, a single bout of flight and dietary lipid composition on pectoralis muscle oxidative enzymes and lipid transporters. Starlings were either unexercised or trained over 2 weeks to fly in a wind tunnel and sampled either immediately following a long flight at the end of this training or after 2 days recovery from this flight. Additionally, they were divided into dietary groups that differed in dietary fatty acid composition (high polyunsaturates versus high monounsaturates) and amount of dietary antioxidant. Trained starlings had elevated (19%) carnitine palmitoyl transferase and elevated (11%) hydroxyacyl-CoA dehydrogenase in pectoralis muscle compared with unexercised controls, but training alone had little effect on lipid transporters. Immediately following a long wind-tunnel flight, starling pectoralis had upregulated lipid transporter mRNA (heart-type fatty acid binding protein, H-FABP, 4.7-fold; fatty acid translocase, 1.9-fold; plasma membrane fatty acid binding protein, 1.6-fold), and upregulated H-FABP protein (68%). Dietary fatty acid composition and the amount of dietary antioxidants had no effect on muscle catabolic enzymes or lipid transporter expression. Our results demonstrate that birds undergo rapid upregulation of catabolic capacity that largely becomes available during flight itself, with minor effects due to training. These effects likely combine with endogenous seasonal changes to create the migratory phenotype observed in the wild.

KEY WORDS: Fatty acid binding protein, Catabolic capacity, Carnitine palmitoyl transferase, Pectoralis muscle, Migration

INTRODUCTION

During long-distance migration, birds undergo drastic remodeling at multiple levels of organization: body composition is altered by impressive changes in body fatness, locomotory muscles increase in size while other organs may decrease in size, and structural and biochemical changes occur within organs to support sustained elevated rates of oxidation. The regulatory mechanisms of such changes are not always well elucidated but can be multiple. For example, seasonal increases to flight muscle mass are known to occur in the absence of exercise (Dietz et al., 1999; Vézina et al., 2007; Price et al., 2011), but exercise (flight) itself also likely plays a regulatory role in muscle size (Dietz et al., 1999; Vézina et al., 2007; Young et al., 2021). Birds thus provide interesting models for examining phenotypic flexibility in exercise physiology, as they undergo acute bouts of exercise in single long-distance flights, undergo chronic exercise training in the form of multiple exercise bouts performed over a migratory season, and prepare for migratory exercise via endogenous seasonal changes.

Within the major flight muscles (pectoralis muscle), lipid oxidative capacity is known to increase during migratory periods, including upregulation of fatty acid transporters and enzymes involved in lipid oxidation (Driedzic et al., 1993; Guglielmo et al., 2002; Lundgren and Kiessling, 1985; Marsh, 1981; McFarlan et al., 2009; Pelsers et al., 1999; Zhang et al., 2015a). This change in muscle biochemistry may be part of an overall increase in oxidative capacity during a period of high energy demand, and/or a specific shift toward lipid catabolic pathways. The mechanisms by which this seasonal upregulation occurs are poorly known, but as in the case of muscle size, they could operate via endogenous circannual cycles (perhaps entrained by environmental cues) and/or as an effect of the increased exercise associated with migration.

Migratory birds have been shown to increase lipid oxidative capacity in pectoralis muscles in response to seasonal photoperiod cues in the absence of migratory flight (Zajac et al., 2011). This includes increased activity of citrate synthase (CS, part of the citric acid cycle), 3-hydroxyacyl-CoA dehydrogenase (HOAD, involved in β-oxidation) and carnitine palmitoyl transferase (CPT, involved in the transport of fatty acyl-CoAs across the mitochondrial membranes) (Zajac et al., 2011). Additionally, transcription of the fatty acid transporters fatty acid translocase (FAT/CD36) and heart-type fatty acid binding protein (H-FABP) was upregulated, resulting in increased protein abundance of at least H-FABP (Zajac et al., 2011) (see also Srivastava et al., 2014; Banerjee and Chaturvedi, 2016). Although substantial, these increases were generally not as large as those observed in the wild for the same species (McFarlan et al., 2009), indicating that the exercise associated with migratory flight may be necessary to induce the full seasonal response. Additionally, other studies of captive and wild birds have suggested that migratory exercise may be necessary to induce the increased oxidative capacity observed in flight muscles of wild migrants (Bishop et al., 1998; Guglielmo et al., 2002; Lundgren and Kiessling, 1986; Pelsers et al., 1999; Price et al., 2010).

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Modification of oxidative and lipid transport capacity in response to exercise may occur over short (acute) or long (training) time scales. In mammals, oxidative enzymes such as CS can increase in response to both acute exercise and training (Kiens et al., 1993; Leek et al., 2001; Siu et al., 2003), although this is not consistently observed (Lawler et al., 1993). Lipid transport is more complicated because of the existence of intracellular stores of lipid transmembrane transporters. Thus, acute exercise can result in intracellular translocation of these transporters without an increase in whole-muscle transporter abundance (Bradley, 2012; Kiens et al., 1997; Bonen et al., 1999; Luiken et al., 2002). Over longer time periods, however, these fat transporters increase in abundance and mRNA expression as a result of training (Bradley, 2012; Bonen et al., 1999; Kiens et al., 1997). It is unknown how most of these transporters change in response to exercise in birds, which differ from mammals in relying heavily on fatty acid oxidation to fuel both rest and exercise.

Dietary lipid composition may also influence oxidative capacity (Dick and Guglielmo, 2019). Transcription of CPT, HOAD, FAT/CD36 (indirectly) and other proteins involved in lipid catabolism can be upregulated by peroxisome proliferator-activated receptor (PPAR) response elements, which require binding of PPAR (Gulick et al., 1994; McClelland, 2004; Sato et al., 2002); PPARs are in turn activated by binding with fatty acids, and the differential ability of various dietary fatty acids to act as ligands for PPAR has been proposed to modulate oxidative enzyme activity in birds (Weber, 2009; DeMoranville et al., 2019). In particular, omega-3 fatty acids have been proposed to induce higher lipid catabolic enzyme activity (Weber, 2009), although this has not always been observed (Price and Guglielmo, 2009; Dick and Guglielmo, 2019), and the response of PPARs to different fatty acids is complex (Hamilton et al., 2018). Additionally, the composition of membrane phospholipids has been hypothesized to affect exercise via their effects on membrane-associated enzymes and transporters (Ayre and Hulbert, 1997; Price et al., 2010; Springer et al., 2011; Valencak et al., 2003; Weber, 2009; Dick and Guglielmo, 2019), while dietary fatty acids have also been proposed to act through their role as signaling molecules (Carter et al., 2020). Despite these multiple potential pathways by which dietary fatty acid composition may affect exercise performance, there are still only a handful of studies that have examined these effects.

In the present study, we examined the effects of acute and chronic exercise on lipid transport and oxidative capacity in the pectoralis muscles of European starlings (*Sturnus vulgaris*). Further, we investigated how lipid transport and oxidative capacity are influenced by dietary lipid composition. We predicted that chronic exercise would result in a training effect, increasing overall oxidative capacity in pectoralis muscles as well as upregulating fatty acid transport gene and protein expression. In contrast, we predicted that an acute bout of exercise would have little effect on fat transporter gene expression, but would increase oxidative enzyme activity. Finally, we predicted that starlings fed a diet enriched with polyunsaturated fatty acids would increase pectoralis oxidative enzyme activity.

MATERIALS AND METHODS

Animals, diet and exercise treatment

European starlings (*Sturnus vulgaris* Linnaeus 1758) are an introduced species in North America, and are migratory in the Great Lakes region (Cabe, 1993). We captured starlings in southern Ontario, Canada, in July and maintained them in indoor aviaries until September, at which point they were randomly assigned to their treatment groups. This study is part of a group of studies on these birds (Hall et al., 2014; Nebel et al., 2012; Price et al., 2011), and complete methodology on husbandry, diets and training can be found in Hall et al. (2014). Briefly, starlings were fed one of four synthetic diets in a 2-factorial design that manipulated the fatty acid composition of the diet and the availability of a potent dietary antioxidant. Diets were isocaloric, had the same macronutrient composition (41% carbohydrate:13% protein:30% fat), and only differed in the relative amounts of plant oils that comprised the dietary fat (olive oil, sunflower oil) and the amount of supplementary vitamin E (5 or 30 IU kg^{-1} diet). Specifically, the four diets included a high monounsaturated diet (MUFA: 72% monounsaturated, 13% polyunsaturated) based on olive oil, and a polyunsaturated diet (PUFA: 68% monounsaturated, 17% polyunsaturated) based on sunflower oil, and these were supplemented with either low or high amounts of vitamin E. Oleic acid (18:1) was the predominant (>97%) MUFA in both diets, whereas linoleic acid (18:2), an omega-6 fatty acid, and α-linolenic acid (18:3), an omega-3 fatty acid, were the predominant (>98%) PUFAs in the two diets.

Birds from these four diet treatments were also assigned to one of three exercise-related treatment groups. Two groups were trained to fly in a wind tunnel at a set wind speed of 12 m s^{-1} with mostly increasing flight durations each day over a 2 week period (day 1 maximum flight time: 10 min, day 2: 10 min, day 3: 20 min, day 4: 30 min, day 5: 30 min, day 6: 45 min, day 7: 60 min, day 8: 90 min, day 9: 30 min, day 10: 120 min, day 11: 180 min, day 12: no flight training, day 13: 60 min, day 14: 30 min). On day 15, birds flew a long flight lasting as long as the birds would voluntarily fly (up to 4 h). One of these groups was sampled immediately following flight ('post-flight' treatment, n=16), allowing us to investigate the acute effects of exercise. The other group was allowed to recover for 2 days before sampling ('trained' treatment, n=40). This 'trained' group was used to investigate the effect of long-term or chronic exercise training, without the confounding effect of recent, acute exercise. A third treatment group consisted of birds that were not flown in the wind tunnel ('untrained', n=59). Flight training of the 56 starlings was accomplished by randomly selecting three birds from a given diet group to start the 2 week flight training every 3 days starting in mid-September. This flight-group selection was stratified so that birds from each of the four diet groups were chosen consecutively, and birds furthest along in their pre-basic I molt were flight trained earliest. The resulting 18 cohorts completed their flight training by late November.

Starlings were euthanized under isoflurane anesthesia by decapitation, and the pectoralis muscle was removed, weighed, frozen in liquid nitrogen, and stored at −80°C until later analysis. Experimental procedures were approved by the University of Western Ontario Animal Care and Use Subcommittee (#2006-011-04).

Enzyme assays

Pectoralis muscle samples (approximately 100 mg) were combined with 9 volumes of a homogenization buffer [20 mmol l^{-1} Na_2HPO_4, 0.5 mmol l^{-1} EDTA, 0.2% defatted BSA, 50% glycerol (Caledon Laboratories, Georgetown, ON, Canada), 0.1% Triton X-100 and Aprotinin at 50 µg ml^{-1}). This was then homogenized 3×10 s on ice, waiting 30 s between bouts. Samples were also sonicated 3×10 s, waiting 30 s between bouts with samples on ice. The sonication power was set to prevent foaming of the samples. The samples were then stored again at −80°C until conducting enzyme assays. All enzyme assays were performed in

duplicate at 39°C in 1 ml reaction volume in disposable polystyrene cuvettes on a Cary 100 Bio Spectrophotometer (Varian Inc., Palo Alto, CA, USA). Assays were run with one reagent omitted (see below), and activity of this control run was subtracted from activity measured in the full assay.

Enzyme assays were modified from Suarez et al. (1986). CPT (EC 2.3.1.21; n=115) was assayed in 50 mmol l^{-1} Tris buffer, pH 8.0, with 10 mmol l^{-1} carnitine (omitted in control runs), 0.15 mmol l^{-1} DTNB, 16 µmol l^{-1} palmitoyl CoA and 10 µl of homogenate diluted 1:5. CS (EC 2.3.3.1; n=88) was assayed in the same Tris buffer, with 0.5 mmol l^{-1} oxaloacetic acid (omitted for control), 0.15 mmol l^{-1} DTNB, 0.25 mmol l^{-1} acetyl CoA and 10 µl of homogenate diluted 1:30. HOAD (EC 1.1.1.35; n=98) was assayed in 50 mmol l^{-1} imidazole buffer, pH 7.4, with 0.2 mmol l^{-1} NADH, 2 mmol l^{-1} EDTA, 0.1 mmol l^{-1} acetoacetyl CoA (omitted for control) and 10 µl of the homogenate diluted 1:10. Lactate dehydrogenase (LDH; EC 1.1.1.27; n=90) was assayed in the same imidazole buffer with 4 mmol l^{-1} pyruvate (omitted for control), 0.3 mmol l^{-1} NADH, 5 mmol l^{-1} DTT and 10 µl homogenate diluted 1:100. Activity was calculated from the change in absorbance: ΔA_{412} for CS and CPT and ΔA_{340} for HOAD and LDH. Enzyme activity is reported in enzyme units (U; µmoles substrate converted per min) and was normalized per gram protein (determined by Bradford assay).

mRNA expression

We measured the mRNA expression (n=91) of a cytosolic fatty acid transporter (H-FABP), two membrane fatty acid transporters (FAT/CD36 and plasma membrane fatty acid binding protein, FABPpm) and two housekeeping genes (actin and GAPDH) using real-time (quantitative) PCR (qPCR) using methods and reagents previously described (McFarlan et al., 2009; Price et al., 2010). Briefly, we extracted RNA from ∼100 mg pectoralis muscle using TRIzol (Invitrogen, Burlington, ON, Canada) in a glass homogenizer. We quantified RNA spectrophotometrically at λ=260 nm in a Tris-EDTA buffer (absorbance of 1 is equal to 40 mg ml^{-1} RNA). Only samples with A_{260}/A_{280}>1.8 were used. RNA samples were then reverse transcribed to create cDNA. We prepared negative controls using water instead of RNA. cDNA was stored at −80°C until qPCR analysis.

For H-FABP, FABPpm, GAPDH and actin, we used qPCR primers previously designed for white-throated sparrows (*Zonotrichia albicollis*; McFarlan et al., 2009) (Table 1). To verify that these primers (supplied by Invitrogen) amplified the intended target genes in starlings, we amplified cDNA with the primers under the same conditions used for qPCR (but omitting SYBR Green), and confirmed the presence of a single band in gel electrophoresis for each gene. Further, we extracted cDNA from that band (QIAquick Gel Extraction Kit, Qiagen Inc., Mississauga, ON, Canada) and had it sequenced at the London Regional Genomics Center at the Robarts Research Institute, University of Western Ontario. For FABPpm and GAPDH, we used additional primer sets to generate sequences before submission to GenBank (Table 1). For FAT/CD36, we used several sets of degenerate and specific primers designed for white-throated sparrows (McFarlan et al., 2009) to produce overlapping sequences for this gene in starlings. We then used this combined sequence to design specific primers to amplify starling FAT/CD36 using qPCR (Table 1). We verified that these primers amplified only FAT/CD36 as described above.

qPCR was performed with a Rotor-Gene 6000 Real-Time Rotary Thermocycler (Corbett Life Science, Concorde, NSW, Australia). The reaction conditions were 1× rxn buffer, 3.5 mmol l^{-1} $MgCl_2$, 0.2 mmol l^{-1} dNTPs, 0.25 µmol l^{-1} primers, 0.75 U Platinum Taq polymerase, 0.7× SYBR Green I, with 1 µl cDNA that had been previously diluted (1:4 in water) in 20 µl reaction volume. Samples were heated at 95°C for 10 min and were then cycled 45 times at 95°C for 10 s, 56°C for 15 s, 72°C for 20 s and 83°C for 0 s. Fluorescence of the samples was measured at the end of the 83°C point of each cycle.

For each gene, samples were run in duplicate, and the cycle threshold of each sample was compared with a calibrator that was present in every run. The calibrator was created from a pool of several starlings' cDNA. Reaction efficiency for each gene was determined using a serial dilution of the calibrator. Expression in each sample was calculated as $\text{Efficiency}^{\Delta Ct}$, where ΔCt is the cycle threshold of the calibrator minus the cycle threshold of the sample. Data are reported as an expression ratio, which we calculated as the expression of each transporter gene divided by the geometric average of the expression of the two housekeeping genes (Vandesompele et al., 2002).

Protein expression

We quantified protein expression of H-FABP in a subset of 28 starlings using western blotting according to methods previously described (Zajac et al., 2011). Briefly, frozen pectoralis muscle samples (∼50 mg) were homogenized in 1 ml of lysis buffer (50 mmol l^{-1} Tris-HCl, 1 mmol l^{-1} EDTA, 2 mmol l^{-1} DTT, 1 mmol l^{-1} PMSF, 1% Triton X-100, pH 7.5), and then sonicated and centrifuged at 1500 ***g*** at 4°C for 15 min. The supernatant was collected and we quantified total protein concentration using Bradford reagent with bovine serum albumin as a standard (BioRad, Mississauga, ON, Canada). Samples were diluted to 1 µg total protein μl^{-1} with 80% Tris-EDTA buffer, and 20% sample buffer (50% sucrose, 7.5% SDS, 62.5 mmol l^{-1} Tris-HCl, 2 mmol l^{-1} EDTA, 3.1% DTT, 0.01% Bromophenol Blue). Samples were heated for 5 min at 95°C and 20 µl of each diluted sample was

Table 1. Primers used for sequencing and quantitative PCR

Gene and primer name	Forward primer (5′–3′)	Reverse primer (5′–3′)	GenBank accession no.
CD36 F1/M1R	CAAAGAGGwCCwTAyACnTA	CTrCAwATnTCAGArGArAArAA	HQ108114
CD36 RT1A/R1R	CATACTGGGAAGGCCACTGT	CTCATTwArCCAmAGwATAGG	
*CD36 RT5A/RT5B	GTGGTTCCTCGTGAAGCATT	TCAGGCCTTCCACGTTATTC	
FABPpm 1A/1B	TTCCAGAGAAGAGCATCATCC	GTCAGTGATGTGCTGCCAGT	HQ108115
*FABPpm RT1A/RT1B	GTGGAAGGAGTTGGCAGCTA	CTCTCCATACAGCCCCATGT	
*H-FABP RT1A/RT1B	AAGACCCAGAGCACCTTCAA	AACAGCGATGTCTCCTTCC	HQ156221
GAPDH 2A/B	GCAGATGCTGGTGCTGAATA	ACAGACACGTTAGGGGTTGG	HQ108116
*GAPDH RT1A/RT1B	CAGCAATGCTTCCTGCACTA	CCTCTGCCATCTCTCCAAAG	
*Actin RT1A/RT1B	CCCTGAAGTACCCCATTGAA	GGGGTGTTGAAGGTCTCAAA	HQ156222

*Primers used for qPCR. CD36 qPCR primers were designed using BLAST (Altschul et al., 1990); all others are from McFarlan et al. (2009).

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electrophoresed on a 15% polyacrylamide gel. Separated proteins were then transferred to polyvinylidene fluoride membranes, and membranes were incubated in a blocking buffer (5% skim milk, 0.05% Tween 20 in TBS) for 1 h at room temperature.

Membranes were then incubated with rabbit primary antibody designed against H-FABP of western sandpipers (*Calidris mauri*) (Guglielmo et al., 2002) for 1 h at room temperature (1:8000 in blocking buffer), washed, and then incubated with goat anti-rabbit IgG conjugated to HRP (1:10,000 in TBS-T) for 1 h at room temperature. After washing, membranes were exposed to a chemiluminescent reagent (Western Lighting Chemiluminescent Reagent Plus, PerkinElmer, Woodbridge, ON, Canada), and imaged digitally with a gel doc. We obtained bands of the expected ∼15 kDa size, determined by comparison to a molecular marker. Bands were quantified using Image J 1.43 software (National Institutes of Health, Bethesda, MD, USA), and optical density was compared with a control sample that was run on every gel.

Statistical analysis

Some samples were not collected for some variables or were lost prior to analysis; the sample size therefore varied among measurements, and we provide the sample size in each figure legend. Following verification of model assumptions, we analyzed enzyme activity, mRNA abundance and protein abundance using a general linear model with diet, exercise treatment and their interaction as fixed factors. When there was a significant effect of exercise treatment, we used Tukey's *post hoc* test to determine differences between groups. Statistical significance was determined at the α=0.05 level. We also determined correlation coefficients for the measured variables. Statistical analyses were conducted using SPSS 11.0 (SPSS, Chicago, IL, USA). mRNA and protein expression ratio units are arbitrary and in figures we scaled results to present fold-changes relative to controls.

RESULTS

Muscle enzyme activity

Diet had no effect on CPT activity (P=0.97), CS activity (P=0.76), HOAD activity (P=0.61) or LDH activity (P=0.82), and there were no significant interactions with exercise treatment (P>0.42 for all enzymes), so diet was removed from further analyses. Exercise training caused a significant increase (19%; $F_{2,112}$=4.3, P=0.015) in CPT activity compared with that in untrained birds (P=0.034; Fig. 1). Immediately following the long bout of flight, birds tended to have even higher CPT activity, although this was not significantly higher than in trained birds (P=0.95) and was only near-significant compared with that in untrained birds (P=0.08, Fig. 1), perhaps because of the smaller sample size. HOAD activity varied among exercise treatments ($F_{2,95}$=4.98, P=0.009), and was near-significantly higher in trained birds (P=0.058), compared with that in untrained birds and significantly (P=0.018; 11%) higher immediately post-flight compared with that in untrained birds (Fig. 1). We detected a significant effect of treatment group on CS activity ($F_{2,95}$=4.98, P=0.046; Fig. 1), but our *post hoc* test did not locate differences between any two groups. There was no significant effect of exercise treatment on LDH activity ($F_{2,87}$=1.68, P=0.192; Fig. 1).

Fatty acid transporter expression

Partial coding sequences were obtained for FAT/CD36 (GenBank accession number HQ108114), FABPpm (HQ108115), H-FABP (HQ156221), GAPDH (HQ108116) and actin (HQ156222). In comparison to the well characterized mouse sequences, the starling

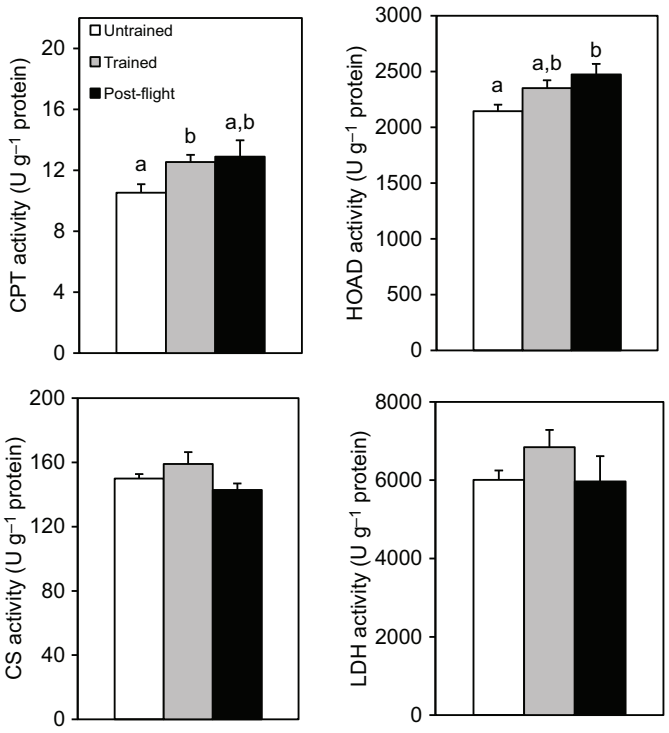

Fig. 1. Enzymatic activity of carnitine palmitoyl transferase (CPT), 3-hydroxyacyl-CoA dehydrogenase (HOAD), citrate synthase (CS) and lactate dehydrogenase (LDH) in pectoralis muscles of European starlings (*Sturnus vulgaris*). Starlings were untrained, or trained for 2 weeks before engaging in a long flight and were then sampled either immediately post-flight ('post-flight') or after 2 days of recovery ('trained'). Data are means+s.e.m. Different letters above bars indicate a significant difference between groups. ANOVA detected a significant effect of exercise treatment on CS activity, but Tukey's *post hoc* test did not find any differences between groups. Sample sizes: CPT untrained n=59, trained n=40, post-flight n=16; HOAD untrained n=48, trained n=35, post-flight n=15; CS untrained n=43, trained n=31, post-flight n=14; LDH untrained n=44, trained n=32, post-flight n=14.

transcripts shared 66–92% identity, and the predicted amino acid sequences shared 58–100% identity (and 76–100% chemical similarity). The primers for actin amplified sequences that aligned most closely with α-actin, although we suspect that both α- and β-actin were measured with our qPCR protocol. Removing actin from the housekeeping gene index had no substantive effect on the mRNA expression results (data not shown).

Diet had no effect on mRNA expression of H-FABP (P=0.47), FAT/CD36 (P=0.24) or FABPpm (P=0.60), and there were no significant interactions with the exercise treatments (P>0.17 for all), so diet was removed from further analyses. Exercise had significant effects on the mRNA expression of all three fatty acid transporters (H-FABP: $F_{2,88}$=16, P<0.0001; FAT/CD36: $F_{2,88}$=5.9, P=0.0036; FABPpm: $F_{2,88}$=10.3, P<0.0001; Fig. 2). For H-FABP protein abundance, two-way ANOVA indicated both a diet effect (P=0.003) and an exercise effect (P=0.009), but further investigation of the diet effect yielded no significant differences among diet groups, so we consider further only the effects of exercise. In trained birds, H-FABP mRNA expression levels were not significantly different from those of the untrained group (P=0.84). However, H-FABP mRNA expression was 4.7-fold higher immediately post-flight than in untrained birds (P<0.001, Fig. 2). This pattern of mRNA expression was reflected in protein expression, although the magnitude of change was not as great

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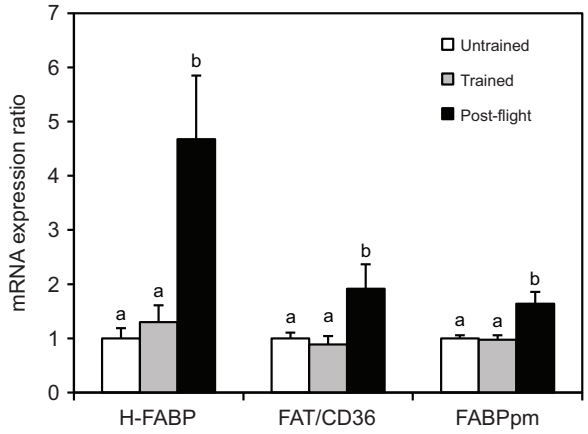

Fig. 2. Expression ratio of fatty acid transporter mRNA in pectoralis muscles of European starlings from the three groups. Heart-type fatty acid binding protein (H-FABP) is a cytosolic fatty acid transporter, while fatty acid translocase (FAT/CD36) and plasma membrane fatty acid binding protein (FABPpm) are located at the plasma membrane and/or mitochondrial membranes. Data are means+s.e.m. Within a gene, different letters above bars indicate a significant difference between groups. Sample sizes: untrained n=45, trained n=31 and post-flight n=15.

(Fig. 3). H-FABP protein expression was 68% higher immediately post-flight when compared with that of untrained birds (P=0.037), but training alone did not increase H-FABP protein abundance (P=0.82). FAT/CD36 mRNA expression was not affected by training (P=0.88), but increased 1.9-fold immediately post-flight (P=0.007, Fig. 2). Similarly, training did not affect FABPpm mRNA expression (P=0.98), but post-flight FABPpm mRNA expression was 1.6-fold higher than that in untrained birds (P<0.001; Fig. 2).

Correlation analyses

Correlation coefficients for enzyme activity, mRNA expression and protein expression for all birds are presented in Table 2. The activities of CPT, CS and HOAD were significantly correlated (P<0.01 for all), and CPT and HOAD activities were significantly correlated with H-FABP protein level (P<0.05). mRNA expression of FAT/CD36 was significantly and highly correlated with mRNA expression of H-FABP (Pearson ρ=0.729, P<0.01) and was correlated with mRNA expression of FABPpm (P<0.01). H-FABP mRNA and protein expression were significantly correlated (ρ=0.468, P<0.01).

Within the post-flight group, we examined correlations between flight duration and enzyme activity or fatty acid transporter expression. Only H-FABP protein expression correlated significantly (ρ=0.706, P=0.022) with flight duration (Fig. 4).

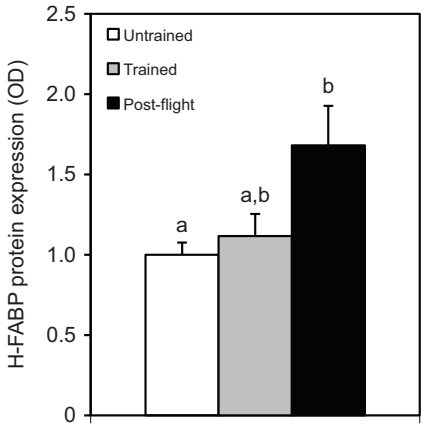

Fig. 3. Expression of H-FABP protein in pectoralis muscle of European starlings from the three groups. Data are means+s.e.m. OD, optical density. Different letters above bars indicate a significant difference between groups. Sample sizes: untrained n=8, trained n=10 and post-flight n=10.

DISCUSSION

Seeking to understand the regulation of oxidative capacity in avian flight muscles, we studied the effects of exercise and dietary fatty acid composition on several lipid oxidative enzymes and transporters in starlings. Our findings suggest that both long-term exercise training and acute bouts of exercise can upregulate important components of the oxidative machinery. In contrast, dietary fatty acid composition had no such effect. In our discussion, we will generally assume that the enzyme activity we measured, with simplified conditions and maximal substrates, does not represent *in vivo* activity, but rather reflects the quantity of a given enzyme present, and thus provides a qualitative index of capacity. Furthermore, we interpret changes in mRNA expression to likely represent qualitative but not necessarily quantitative increases in protein abundance. Indeed, we found an increase in H-FABP mRNA expression of 470% with acute exercise, while H-FABP protein expression increased only 68%, confirming qualitative but not quantitative agreement in this case. Still, we note that in other cases mRNA expression could increase without a corresponding qualitative increase in protein abundance.

Effects of training and acute exercise on enzyme activity and lipid transporter expression

We found evidence for both acute and chronic regulation of various aspects of oxidative metabolism in response to exercise in starlings. Long-term training resulted in upregulation of CPT, a key enzyme which acts to allow the transport of fatty acids across mitochondrial membranes. Additionally, HOAD activity was non-significantly increased by training, but the combination of training and acute exercise resulted in HOAD upregulation compared with that of untrained birds. These findings are supported by the observation that neither CPT nor HOAD activity was significantly affected by flight time; both appear to be upregulated primarily by chronic exercise stimulation but not much by acute stimulation. Zhang et al. (2015b) also found no significant effects of acute exercise on CPT or HOAD activity in house sparrows (*Passer domesticus*), although they did find an increase in CS activity.

In contrast to oxidative enzymes, the lipid transporters we measured – H-FABP, FABPpm, FAT/CD36 – were not affected by training (measured by mRNA expression), but their mRNA expression and protein expression (at least for H-FABP) were upregulated acutely during flight. H-FABP protein expression was also positively correlated with flight time, giving further support to the idea that H-FABP is acutely regulated by exercise, although this correlation could alternatively indicate that birds with higher H-FABP protein expression were simply able to fly longer in the tunnel. H-FABP was also upregulated in a study of house sparrows following acute exercise (Zhang et al., 2015b). However, this finding differs from a recent study of starlings following a similar training protocol, in which trained (but not acutely exercised) birds showed significantly increased expression of FAT/CD36 in the pectoralis muscle (DeMoranville et al., 2020). It is unclear why these results differ given the similarities of species and exercise protocols.

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Table 2. Pearson correlation coefficients for enzyme activity, and mRNA and protein expression

	Enzyme activity			mRNA expression			Protein expression
	CS	LDH	HOAD	FAT/CD36	FABPpm	H-FABP	H-FABP
CPT	0.478** (88)	−0.082 (90)	0.448** (98)	0.051 (92)	0.073 (92)	0.040 (93)	0.415* (31)
CS		−0.015 (88)	0.573** (87)	0.175 (81)	0.191 (81)	0.160 (82)	−0.007 (29)
LDH			−0.125 (89)	−0.149 (83)	−0.155 (83)	−0.136 (84)	−0.015 (29)
HOAD				0.127 (86)	0.135 (86)	0.169 (87)	0.431* (30)
FAT/CD36					0.436** (92)	0.729** (92)	0.635** (30)
FABPpm						0.331** (92)	0.308 (30)
H-FABP (mRNA)							0.468** (30)

CPT, carnitine palmitoyl transferase; CS, citrate synthase; LDH, lactate dehydrogenase; HOAD, 3-hydroxyacyl-CoA dehydrogenase; FAT/CD36, fatty acid translocase; FABPpm, plasma membrane fatty acid binding protein; H-FABP, heart-type fatty acid binding protein. Sample sizes are in parentheses. *P<0.05, **P<0.01.

In mammals, exercise is known to stimulate increased lipid oxidative capacity in muscles via transcriptional and non-transcriptional mechanisms (McClelland, 2004). Acute exercise can induce translocation of FAT/CD36 from intracellular pools to the sarcolemmal membrane (Luiken et al., 2002), while chronic exercise can result in transcriptional upregulation of FAT/CD36 (Bonen et al., 1999) and increased protein abundance of both FAT/CD36 (Bonen et al., 1999) and FABPpm (Kiens et al., 1997). Our results in starlings gave no indication of an effect of chronic exercise on any of these lipid transporters. This is distinct from mammals and locusts, in which H-FABP is thought to be a stable protein and both the protein and mRNA have long half-lives (Carey et al., 1994; Zhang and Haunerland, 1998). This difference between our results and previous findings may derive from the differences between birds and mammals with regard to fuel use; birds rely extensively on fat to fuel most activities (McWilliams et al., 2004), whereas mammals rely on fat only at low intensity activity (McClelland, 2004), such that, in mammals, increasing the use of fat can be beneficial to increasing endurance performance. Alternatively, our focus on mRNA may have hampered our ability to observe changes in lipid transporters at the protein level. We observed no significant rise in H-FABP protein as a result of long-term training, but the remaining transporters (FAT/CD36 and FABPpm) were measured only at the mRNA level. Although mRNA of these transporters subsided by 48 h after intense exercise, protein levels of these transporters may nonetheless have been elevated at the 48 h post-exercise time point.

The effect of acute, but not chronic exercise, on fatty acid transporters may also explain the lack of exercise training exhibited by barnacle geese prior to migration (Portugal et al., 2012). Our data suggest that training may not be necessary to induce upregulation of these major components of the lipid oxidative pathway, because they can be acutely upregulated during flight. Additionally, our results have importance for understanding other types of seasonal changes to avian muscle phenotypes. Some non-migrant birds, for example, have elevated H-FABP in association with their higher summit metabolism during the wintering period (Liknes et al., 2014). Our findings suggest that these seasonal changes may reflect not only long-term acclimation but also recent thermoregulatory activity. It is worth remembering, however, that we have not measured oxidative capacity nor lipid transport capacity at other levels of biological organization. It is possible that at the level of the mitochondria or whole muscle, one might observe different effects.

Our results differ from those we found previously in white-crowned sparrows (*Zonotrichia leucophrys*) that were stimulated to fly back and forth in small cages (Price et al., 2010). Those sparrows showed little effect of exercise on catabolic enzyme activity or fatty acid transporter expression in the pectoralis muscle. This could reflect differences in species, but we suspect that the difference in experimental protocols is the principal driver. The wind tunnel flights by starlings in the current study likely approximate wild flight exercise much better than the burst flight in cages of the previous study.

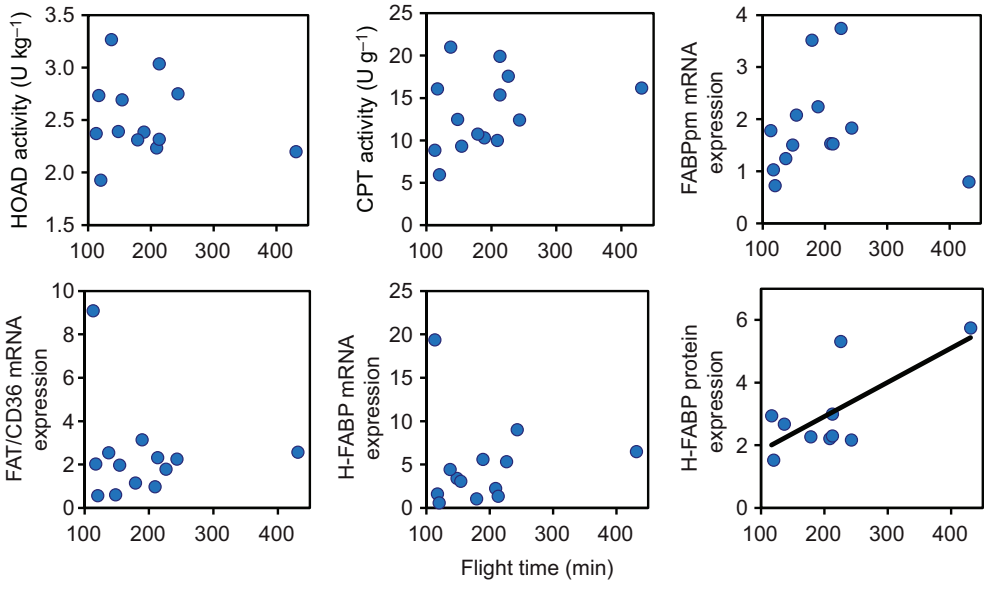

Fig. 4. Correlation of post-flight enzymatic activity (HOAD, CPT), mRNA expression (FABPpm, FAT/CD36, H-FABP) and protein expression (H-FABP) with flight time in European starlings. Starlings were trained for 2 weeks and then flown for a long-duration flight.

It is unclear why some components of the lipid oxidative pathway are upregulated only in response to acute exercise, while others are only upregulated in response to chronic exercise. Our correlation analysis demonstrated that, overall, these components are in fact correlated. This may be explained by the fact that for both CPT and HOAD, there were non-significant trends toward higher activity after a flight bout compared with levels in trained birds.

Effects of dietary fatty acid composition on catabolic enzyme activity

Dietary fatty acid composition can affect various measures of exercise performance (Ayre and Hulbert, 1997; McKenzie et al., 1998; Pierce et al., 2004; Price and Guglielmo, 2009; but see Dick and Guglielmo, 2019). These effects might occur by one or more mechanisms, considering the roles of fatty acids as energy substrates, membrane components and signaling molecules (Price, 2010). Membrane-bound enzymes (such as CPT) could be affected by fatty acids that are incorporated into membranes, and enzyme activity could also be affected by transcriptional upregulation caused by fatty acids interacting with PPAR. A study in quail demonstrated that dietary fatty acid composition could affect the enzyme activity of CS, CPT, HOAD and cytochrome *c* oxidase in the pectoralis muscle (Nagahuedi et al., 2009). This effect was not always simple, with related omega-3 fatty acids having equivalent, additive or cancelling effects, depending on the enzyme (Nagahuedi et al., 2009). A study of the pectoralis muscle of white-throated sparrows failed to find an effect of dietary fatty acid composition on enzyme activity of CPT, HOAD or LDH, although there was a subtle effect on CS (Price and Guglielmo, 2009). In yellow-rumped warblers, *Setophaga coronata*, there was a small effect of dietary fatty acid composition on CPT, HOAD, CS and LDH, but no effect on performance (Dick and Guglielmo, 2019). In the current study, we found no effect of dietary fatty acid composition on enzyme activity of starling pectoralis muscles. This finding largely agrees with the results from a study on starlings using similar diets (DeMoranville et al., 2020), which demonstrated only subtle changes to oxidative gene expression due to dietary lipids. Overall, these findings do not support the hypothesis that dietary fatty acids interact differentially with PPARs to affect enzyme activity, although this result may be specific to the particular species and diets used here.

Summary of findings

We found that flight training increased the enzyme activity of CPT and HOAD in starling pectoralis muscles, while acute exercise induced increases in the mRNA and protein expression of fatty acid transporters. These results support previous suggestions that the elevated lipid transport and oxidative capacities observed in wild migrants are due to a combination of endogenous rhythms and flight exercise (Guglielmo et al., 2002; Lundgren and Kiessling, 1986; Pelsers et al., 1999; Zajac et al., 2011). Future studies should seek to quantify how much phenotypic flexibility is available to long-distance migrants via acute in-flight upregulation of oxidative capacity and, in comparison, how much pre-migratory seasonal upregulation of oxidative capacity may be required to achieve typical migratory flight distances. Future studies could also seek to connect our results with these other levels of biological organization, such as mitochondrial or muscle oxidative capacity. In our study, dietary fatty acid composition had no effect on the activity of these enzymes or on the expression of lipid transporters.

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Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: U.B., S.R.M., C.G.G.; Formal analysis: E.R.P.; Investigation: E.R.P., U.B., S.R.M., M.L.B., L.A.L., A.R.G., C.G.G.; Resources: S.R.M., C.G.G.; Data curation: E.R.P.; Writing - original draft: E.R.P.; Writing - review & editing: E.R.P., U.B., S.R.M., M.L.B., L.A.L., A.R.G., C.G.G.; Visualization: E.R.P.; Supervision: U.B., S.R.M., C.G.G.; Project administration: S.R.M., C.G.G.; Funding acquisition: S.R.M., C.G.G.

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