

## Seasonal upregulation of fatty acid transporters in flight muscles of migratory white-throated sparrows (*Zonotrichia albicollis*)

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### SUMMARY

Endurance flights of birds, some known to last several days, can only be sustained by high rates of fatty acid uptake by flight muscles. Previous research in migratory shorebirds indicates that this is made possible in part by very high concentrations of cytosolic heart-type fatty acid binding protein (H-FABP), which is substantially upregulated during migratory seasons. We investigated if H-FABP and other components of muscle fatty acid transport also increase during these seasons in a passerine species, the white-throated sparrow (*Zonotrichia albicollis*). Fatty acid translocase (FAT/CD36) and plasma-membrane fatty acid binding protein (FABPpm) are well characterized mammalian proteins that facilitate transport of fatty acid through the muscle membrane, and in this study they were identified for the first time in birds. We used quantitative PCR to measure mRNA of FAT/CD36, FABPpm and H-FABP and immunoblotting to measure protein expression of FABPpm and H-FABP in the pectoralis muscles of sparrows captured in migratory (spring, fall) and non-migratory (winter) seasons. During migratory seasons, mRNA expression of these genes increased 70–1000% above wintering levels, while protein expression of H-FABP and FABPpm increased 43% and 110% above wintering levels. Activities of key metabolic enzymes, 3-hydroxyacyl-CoA-dehydrogenase (HOAD), carnitine palmitoyl transferase II (CPT II), and citrate synthase (CS) also increased (90–110%) in pectoralis muscles of migrant birds. These results support the hypothesis that enhanced protein-mediated transport of fatty acids from the circulation into muscle is a key component of the changes in muscle biochemistry required for migration in birds.

Supplementary material available online at <http://jeb.biologists.org/cgi/content/full/212/18/2934/DC1>

Key words: bird, lipid, exercise, migration, oxidative enzymes, metabolism.

### INTRODUCTION

Migratory flight in birds is an impressive feat of endurance exercise in nature. Recent evidence demonstrates that species such as the bar-tailed godwit (*Limosa lapponica* Linnaeus) and probably other shorebirds, can fly non-stop for as long as 9–10 days to cross geographic barriers such as the Pacific Ocean (Gill et al., 2005). During these flights birds maintain a very high rate of oxygen consumption which has been estimated to be at least twice the maximal oxygen consumption ( $\dot{V}_{O_{2max}}$ ) of a typical running mammal of similar size, and approximately 65–85% of the bird's  $\dot{V}_{O_{2max}}$  (Butler et al., 1997; Guglielmo et al., 2002). Moreover, migratory flight is fueled almost exclusively by the oxidation of exogenous fatty acids delivered by the circulation from adipose tissue (Rothe et al., 1987; Jenni and Jenni-Eiermann, 1998; McWilliams et al., 2004). This contrasts with mammalian high-intensity exercise, which is fuelled primarily by intramuscular carbohydrate stores (glycogen), and only switches to exogenous fatty acids at low exercise intensities (McClelland, 2004).

A variety of inter- and intraspecific studies have demonstrated that high activities of enzymes involved in fatty acid oxidation are associated with long-distance migration of birds (Lundgren and Kiessling, 1985; Driedzic et al., 1993; Guglielmo et al., 2002; Mailliet and Weber, 2006). However, in exercising mammals, the most limiting step in the utilization of adipose-derived fatty acids appears to be uptake across the muscle membrane (Vock et al., 1996; Hajri and Abumrad, 2002; McClelland, 2004). In previous studies of western sandpipers (*Calidris mauri* Cabanis) we demonstrated that

heart-type fatty acid binding protein (H-FABP; also known as FABPc and FABP3), a key protein required for high rates of uptake of exogenous fatty acid, was one of the most abundant cytosolic proteins in the flight muscles and heart, and several-fold more abundant than measured in muscles of any other vertebrate species (Guglielmo et al., 1998). During migration, when the demand for exogenous fatty acid (FA) is greatest, flight muscle H-FABP concentration increased by 70% (Pelters et al., 1999; Guglielmo et al., 2002). Pelters et al. (Pelters et al., 1999) observed a similar seasonal increase of H-FABP in barnacle geese (*Branta leucopsis* Bechstein). Although not directly involved in the uptake of FA across cell membranes, H-FABP may serve as an intracellular sink for FA uptake, and is probably required to maintain flux by shuttling FA away from the cell membrane (Glatz et al., 2002). These data suggest that enhanced fatty acid uptake capacity could be a key factor that makes migratory endurance flight possible (Guglielmo et al., 2002; McWilliams et al., 2004).

It is now widely accepted that the transfer of fatty acids across the plasma membrane of muscle is mostly a protein-mediated process (Bonen et al., 2007a). In mammals, the majority of fatty acid uptake across the membrane has been attributed to two proteins; fatty acid translocase (FAT/CD36) and plasma-membrane fatty acid binding protein (FABPpm) (Bonen et al., 2007a). Up to 75% of protein-mediated fatty acid uptake into muscle tissue is facilitated through FAT/CD36, as evidenced by use of the FAT/CD36 specific inhibitor, sulfo-*N*-succinimidyl oleate (SSO) (Luiken et al., 1999). Based on our previous findings with cytosolic

H-FABP we hypothesized that, if birds had similar membrane fatty acid transporters to mammals, seasonal increases in their expression could be crucial to migration (Guglielmo et al., 2002; McWilliams et al., 2004)

English sparrow (*Passer domesticus* Linnaeus) muscle incubated with SSO *in vitro* was previously shown to have a 64% decrease in fatty acid uptake, which indicates a FAT/CD36-like protein in birds (Sweazea and Braun, 2006). However, no study has yet characterized an avian homologue of FAT/CD36 or FABPpm. Furthermore, these proteins have not been investigated in any wild species where variable demand of fatty acid transport into muscle could be critical to physiological performance. Therefore, we hypothesized, first, that migratory birds have genetic homologues of FAT/CD36 and FABPpm that function similarly to the well characterized mammal counterparts. Second, using white-throated sparrows (*Zonotrichia albicollis* Gmelin) we hypothesized that birds would increase expression of these proteins, as well as H-FABP, during migratory seasons. White-throated sparrows were chosen as a model for avian migration in this intraspecific study because migration physiology of this genus has been widely studied, and they are members of the order Passeriformes, which is highly divergent from Charadriiformes and Anseriformes, the subject of previous studies by our lab and others. Similar increases in H-FABP in three highly divergent bird species may indicate that seasonal upregulation of these proteins is a conserved mechanism among migratory birds.

## MATERIALS AND METHODS

### Animal collection

The white-throated sparrow is a 25–30 g passerine bird that breeds in the boreal forests of northern Ontario and Quebec, and migrates as far south as the southern Atlantic coast and Gulf of Mexico for the winter months. The journey between breeding grounds and wintering grounds (3000–5000 km) is made in several nocturnal flights, stopping for poor weather or refuelling (Falls and Kampuchean, 1994). White-throated sparrows were captured by mist-netting in both migratory and non-migratory (wintering) seasons. We captured wintering sparrows between 3rd and 5th February 2006 ( $N=3$ ; two female, one unknown; body mass  $27.8 \pm 1.5$  g), and 8th and 11th January 2007 ( $N=17$ ; 12 female, three male, two unknown; body mass  $28.4 \pm 0.6$  g) near Stoneville, Mississippi at the Delta Experimental Forest ( $33^{\circ}27'34''N$ ,  $90^{\circ}54'8''W$ ), which is managed by the United States Department of Agriculture. We caught migratory sparrows on private land approximately 10 km north of Long Point Provincial Park, Ontario ( $42^{\circ}38'20''N$ ,  $80^{\circ}34'21''W$ ). We captured 19 sparrows (two male, 17 female; body mass  $28.4 \pm 0.5$  g) during spring migration, between 10th and 26th April 2006; and 38 sparrows (18 male, 17 female, three unknown of which 22 were juvenile, 13 adult and three unknown; body mass  $24.7 \pm 0.3$  g) during fall migration, from 3rd October to 23rd October 2006. More birds were captured in the fall for the purpose of examining age effects. It is notoriously difficult to determine the sex of white-throated sparrows by plumage alone (Caldwell and Mills, 2006), therefore, sex was determined post-mortem in the laboratory, and this led to disproportionate capture of males and females in two of the three seasons. We were not able to confidently assign the sex of six birds due to the removal of organs during field dissection. Our study followed the Canadian Council on Animal Care guidelines and was approved by the University of Western Ontario Council on Animal Care and the Animal Use Subcommittee (protocol #2005-060-08). A scientific collection permit was granted by the Canadian Wildlife Service (permit #CA

0168), and the United States Fish and Wildlife Scientific Collection Permit (MB758364-1) to Dr Frank Moore, from the University of Southern Mississippi.

Immediately after capture, sparrows were weighed to the nearest 0.05 g, anesthetized with an inhaled anaesthetic (Isoflurane, Abbott Laboratories, St. Laurent, QC, Canada) and killed by cervical dislocation. A 1–2 g sample from each pectoral muscle was removed, placed in a cryotube and immediately frozen in liquid nitrogen. After transport to the lab, all tissue samples were stored at  $-80^{\circ}C$  until analysis. During later dissection, birds were sexed by visual inspection of gonads, and in the fall aged by the degree of skull ossification (Pyle, 1997).

### Avian genetic homologues

The well characterized human protein sequences for FAT/CD36, FABPpm and H-FABP (GenBank Acc. No: NM\_001001547; NM\_002080.2; NM\_004102.3, respectively) were BLAST searched against a chicken database (Hubbard et al., 2007). The resulting homologous sequences were aligned across multiple vertebrate species and degenerate primers for PCR were designed from conserved regions of each alignment. Degenerate primers were designed using Primer3 software (Rozen and Skaletsky, 2000) before being commercially manufactured (Invitrogen, San Diego, CA, USA). Resulting PCR products were sequenced to confirm their identity (Applied Biosystems 3730 Analyzer, Robarts Research Institute, London, ON, Canada) and new primers were designed for use in real-time PCR (supplementary material Table S1).

### RNA isolation and reverse transcription

Frozen muscle tissue (50–100 mg) was ground with a mortar and pestle cooled in liquid nitrogen, and total RNA was extracted using TRIzol in a glass homogenizer following the manufacturer's protocol (Invitrogen, Burlington, ON, Canada), which is an adaptation of Chomczynski's method (Chomczynski and Sacchi, 1987). The final RNA pellet was stored at  $-20^{\circ}C$  until analysis. All RNA samples were eluted in sterile water, heated to  $55^{\circ}C$  for 10 min (to aid in dissolution of the pellet) and quantified spectrophotometrically in Tris-EDTA buffer ( $10 \text{ mmol l}^{-1}$  Tris, pH 8,  $1 \text{ mmol l}^{-1}$  EDTA) at  $\lambda_{260}$  and only samples with  $\lambda_{260}/\lambda_{280}$  values  $>1.8$  were used. To remove any contaminating DNA,  $5 \mu\text{g}$  of total RNA was digested with 2 i.u. DNase I (New England Biolabs, London, ON, Canada) incubated at  $37^{\circ}C$  for 15 min and then heat inactivated at  $75^{\circ}C$  for 10 min. Reverse transcription was carried out immediately using  $0.5 \mu\text{g}$  of total RNA,  $0.5 \mu\text{g}$  oligo(dT)<sub>12-18</sub> primer,  $0.5 \mu\text{mol l}^{-1}$  dNTPs,  $1 \times$  first strand buffer ( $250 \text{ mmol l}^{-1}$  Tris, pH 8.3,  $375 \text{ mmol l}^{-1}$  KCl,  $15 \text{ mmol l}^{-1}$  MgCl<sub>2</sub>),  $10 \text{ mmol l}^{-1}$  dithiothreitol (DTT),  $1 \mu\text{l}$  RNaseOUT, and 200 i.u. SuperScript II reverse transcriptase (Invitrogen), in a  $20 \mu\text{l}$  volume for 90 min at  $42^{\circ}C$  followed by termination for 15 min at  $70^{\circ}C$ . The resulting cDNA solutions were stored at  $-80^{\circ}C$  until analysis. Negative controls were prepared using sterile water and a no-template TRIzol preparation, which were carried through to real-time PCR.

### Real-time PCR

Each cDNA sample was diluted 1:5 with sterile water before being used in real-time PCR performed with a Rotor-Gene 6000 Real-Time Rotary Thermocycler (Corbett Life Science, Montreal Biotech, Montreal, QC, Canada). Reaction chemistries varied among primer pairs, and optimizing the conditions for each pair was facilitated by the use of a homemade SYBR-Green I mastermix adapted from other studies (Karsai et al., 2002). The

reaction conditions for FAT/CD36 and FABPpm were  $1\times$  reaction buffer ( $20\text{ mmol l}^{-1}$  Tris,  $50\text{ mmol l}^{-1}$  KCl, pH 8.4),  $3.5\text{ mmol l}^{-1}$ ,  $\text{MgCl}_2$  ( $4\text{ mmol l}^{-1}$   $\text{MgCl}_2$  for H-FABP, GAPDH and  $\beta$ -actin),  $0.2\text{ mmol l}^{-1}$  dNTPs,  $0.25\text{ }\mu\text{mol l}^{-1}$  primers, 0.75 i.u. Platinum Taq polymerase,  $0.7\times$  SYBR-Green I (Invitrogen) in  $10\text{ }\mu\text{l}$  volume. Cycling conditions did not vary between primer pairs, which allowed different primers to be run at the same time (initial  $95^\circ\text{C}$  for 7 min followed by 50 cycles of  $56^\circ\text{C}$  for 20 s,  $72^\circ\text{C}$  for 15 s,  $83^\circ\text{C}$  for 4 s,  $94^\circ\text{C}$  for 15 s, and finally a melt curve analysis from  $72$ – $99^\circ\text{C}$ ). Each gene of interest was assayed in a separate tube for each individual bird, in duplicate. Fluorescence of SYBR-Green I in the samples was measured after the extension phase of each cycle at  $83^\circ\text{C}$  with an excitation at 470 nm and detection at 510 nm. No calibration dye (i.e. ROX<sup>TM</sup>) is required when using the Rotor-Gene 6000 Thermocycler as the centrifugal design rotates samples past the excitation/detection probe several times per second during the data acquisition phase.

#### Immunoblots

Crude membrane proteins were prepared for immunoblotting using previously established protocols for mammalian muscle (Luiken et al., 2002). Briefly, 50–80 mg of frozen sparrow muscle was homogenized in 2 ml of homogenization buffer A ( $210\text{ mmol l}^{-1}$  sucrose,  $2\text{ mmol l}^{-1}$  EGTA,  $40\text{ mmol l}^{-1}$  NaCl,  $30\text{ mmol l}^{-1}$  Hepes,  $5\text{ mmol l}^{-1}$  EDTA,  $2\text{ mmol l}^{-1}$  PMSF and 0.6% DMSO) with a Polytron homogenizer ( $2\times 10\text{ s}$  bursts). Another 2 ml of homogenization buffer A was added, along with 3 ml buffer 1 ( $1.167\text{ mol l}^{-1}$  KCl,  $58.3\text{ mol l}^{-1}$  tetra-sodium pyrophosphate), and the homogenate was vortexed and then ultracentrifuged at  $171,500\text{ g}$  at  $4^\circ\text{C}$  for 75 min. The supernatant was discarded and the pellet containing the membrane fractions was resuspended in  $600\text{ }\mu\text{l}$  buffer 2 ( $10\text{ mmol l}^{-1}$  Tris,  $1\text{ mmol l}^{-1}$  EDTA, pH 7.4) with a Polytron homogenizer. To disrupt the membranous structures,  $200\text{ }\mu\text{l}$  of 16% SDS was added, followed by vortexing of the mixing and centrifugation at  $2500\text{ g}$  for 15 min at room temperature. The supernatant, which contained the solubilized membrane protein fraction was stored at  $-80^\circ\text{C}$  until protein concentration was determined using bicinchoninic acid solution (Sigma, Oakville, ON, Canada) (Smith et al., 1985).

Cytosolic proteins were isolated by homogenizing 50–80 mg sparrow muscle in 1 ml of cytosolic homogenization buffer B [ $50\text{ mmol l}^{-1}$  Tris (pH 7.5),  $1\text{ mmol l}^{-1}$  EDTA,  $1\text{ mmol l}^{-1}$  EGTA,  $50\text{ mmol l}^{-1}$  NaF,  $10\text{ mmol l}^{-1}$  sodium  $\beta$ -glycerol phosphate,  $5\text{ mmol l}^{-1}$  sodium pyrophosphate,  $2\text{ mmol l}^{-1}$  DTT,  $1\text{ mmol l}^{-1}$  sodium orthovanadate,  $1\text{ mmol l}^{-1}$  PMSF, and  $10\text{ }\mu\text{g ml}^{-1}$  of protein inhibitors apoprotinin, leupeptin and pepstatin A] with a Polytron homogenizer ( $2\times 10\text{ s}$  bursts). Homogenates were centrifuged at  $15,000\text{ g}$  at  $4^\circ\text{C}$  for 30 min, and the protein content of the supernatant was determined by the Bradford assay (Bradford, 1976).

All protein samples were diluted to  $1\text{ }\mu\text{g }\mu\text{l}^{-1}$  total protein, mixed 5:1 with sample buffer [ $50\%$  sucrose,  $7.5\%$  SDS,  $62.5\text{ mmol l}^{-1}$  Tris (pH 6.8),  $2\text{ mmol l}^{-1}$  EDTA (pH 7.5),  $3.1\%$  DTT,  $0.01\%$  Bromophenol Blue], heated to  $95^\circ\text{C}$  for 5 min and  $30\text{ }\mu\text{g}$  of total protein was loaded for SDS-PAGE at 150 V for 1 h using a Mini-Protean 3 Electrophoresis Unit (Bio-Rad, Mississauga, ON, Canada) in running buffer ( $25\text{ mmol l}^{-1}$  Tris,  $192\text{ mmol l}^{-1}$  glycine,  $0.1\%$  SDS). Separated proteins were transferred to PVDF membranes using transfer buffer ( $25\text{ mmol l}^{-1}$  Tris,  $192\text{ mmol l}^{-1}$  glycine,  $20\%$  methanol) at 100 V for 1 h. For FABPpm, membranes were blocked with  $7.5\%$  BSA in TBS-T ( $20\text{ mmol l}^{-1}$  Tris,  $137\text{ mmol l}^{-1}$  NaCl,  $100\text{ mmol l}^{-1}$  HCl,  $0.01\%$  Tween 20, pH 7.5) for 1 h at  $22^\circ\text{C}$ , then with the primary antibody [anti-FABPpm 1:30,000, kindly provided

by Dr Calles-Escandon; anti-H-FABP 1:8000 (Guglielmo et al., 2002)] overnight at  $4^\circ\text{C}$ , washed for 10 min three times with TBS-T, and incubated with a secondary antibody for 1 h at  $22^\circ\text{C}$ . All secondary antibodies (NA934V 1:3000 for FABPpm available from Amersham Biosciences, Baie d'Urfé, Québec, Canada; sc-2004 for 1:5000 for H-FABP available from Santa Cruz Biotech, CA, USA) were conjugated to horseradish peroxidase, which was detected using Perkin Elmer Western Lighting Chemiluminescent Reagent Plus (Perkin Elmer, Woodbridge, ON, Canada) and a Syngene ChemiGenius2 Bio-imaging System (Synoptics, Frederick, MD, USA) running Syngene GeneSnap software (version 6.08). Bands were then quantified using Syngene GeneTools software (version 3.07), and optical density (OD) for each band was quantified relative to a control run on all gels. All attempts to detect FAT/CD36 with available antibodies failed. FAT/CD36 is a heavily glycosylated protein, and this post-transcriptional modification is possibly different in birds, making it difficult to detect using mammal-derived antibodies.

#### Enzyme assays

To determine oxidative capacity of muscles, maximal enzyme activities of citrate synthase (CS), 3-hydroxyacyl-CoA-dehydrogenase (HOAD) and carnitine palmitoyl transferase 2 (CPT II) were measured as described previously (Woeltje et al., 1987; Guglielmo et al., 2002) using a UV/Vis spectrophotometer (Cary 100, Varian, Mississauga, ON, Canada) with a digitally controlled water bath to maintain reaction temperature at  $39^\circ\text{C}$ . Maximal activity of lactate dehydrogenase (LDH) was measured similarly, using  $0.15\text{ mmol l}^{-1}$  NADH in a  $50\text{ mmol l}^{-1}$  imidazole buffer solution (pH 7.4) with  $5\text{ mmol l}^{-1}$  DTT and adding pyruvate to a final concentration of  $4\text{ mmol l}^{-1}$  to initiate the reaction (McCullagh et al., 1996).

#### Data analysis

RT-PCR reaction efficiencies of both GAPDH and  $\beta$ -actin were 1.88, and when their  $\Delta\text{Ct}$  was plotted against a logarithmic range of cDNA concentrations the resulting slope was not statistically different from zero (least squares linear regression;  $R^2=0.0997$ ,  $P>0.1$ ; data not shown), which validates their use together in a housekeeping gene index (Livak and Schmittgen, 2001; Crnkovic-Mertens et al., 2007). The housekeeping gene index was created using the geometric average of  $\Delta\text{Ct}$  values for GAPDH and  $\beta$ -actin of each individual bird (Vandesompele et al., 2002; Pfaffl et al., 2004; Peters et al., 2007). The expression of the target genes (FAT/CD36, FABPpm and H-FABP) was then calculated as a relative value to the housekeeping gene index for each individual bird (Eqn 1), where  $E$  is efficiency of gene amplification,  $\Delta\text{Ct}$  is the difference in Ct values between a calibrator and an individual bird for the gene of interest ( $\Delta\text{Ct}_T$ ) and housekeeping gene ( $\Delta\text{Ct}_H$ ). The distribution of expression ratios for each gene were skewed and variances were unequal, therefore, non-parametric methods were used for all RT-PCR data.

$$\text{Expression ratio} = (E_{\text{target}})^{\Delta\text{Ct}_T} / (E_{\text{housekeeper}})^{\Delta\text{Ct}_H} \quad (1)$$

Except for FABPpm, all enzyme and immunoblot data were tested with parametric methods. All results were first tested for differences between sexes and ages in fall migrants only, then spring and fall migrants. If no statistical difference was observable between spring and fall migrations, these seasons were pooled together to increase statistical power when testing against winter (non-migration). Statistical analyses were performed using SPSS v14 software (SPSS).

## RESULTS

### RNA sequences

Total RNA extracted from pectoral muscles was correlated with the amount of muscle tissue used in the TRIzol extraction (Spearman's  $\rho=0.615$ ,  $P<0.001$ ). There was no significant difference in total RNA per milligram of tissue between spring and fall birds (ANCOVA  $F=3.338$ ,  $P=0.073$ ), and when grouped together, migratory birds had more total RNA per mg muscle tissue than wintering birds ( $F=14.999$ ,  $P<0.001$ ). Spring and fall birds independently also had significantly more total RNA per milligram of tissue than wintering birds ( $F=8.469$ ,  $P=0.006$ , and  $F=25.273$ ,  $P<0.001$ , respectively). Total RNA per milligram of tissue did not vary with age, sex or body mass ( $P>0.1$ ).

Partial sequences of reverse-transcribed mRNA (i.e. cDNA) for FAT/CD36 (GenBank accession number EU556709), FABPpm (EU556710), H-FABP (EU556711), TATAbp (EU556708), GAPDH (EU556707) and  $\beta$ -actin (EU556706) were obtained from white-throated sparrow pectoralis muscle tissue. The cDNA sequence for white-throated sparrow transcripts share 60–80% identity with well characterized mouse sequences, and the predicted peptide sequences share 65–100% identity (and 77–100% similarity). When compared with a previously published western sandpiper H-FABP protein sequence (Guglielmo et al., 1998), the predicted white-throated sparrow H-FABP peptide had 92% identity (and 97% similarity). Each of these sequences shares high homology across species, which indicates the protein functions are highly conserved and there may be a selective pressure to maintain their function.

### Transporter mRNA variation

The expression of FAT/CD36 mRNA in pectoral muscle was 100% higher in spring migrants than in wintering birds (Mann–Whitney  $U=66$ ,  $P<0.001$ ) and fall migrants had an additional 170% higher expression than spring migrants (Mann–Whitney  $U=155$ ,  $P<0.001$ ) (Fig. 1A). The expression of FABPpm mRNA exhibited similar trends to those seen for FAT/CD36 mRNA. Spring birds had 80% higher expression than wintering birds (Mann–Whitney  $U=70$ ,  $P=0.002$ ) and fall migrants had an additional 70% higher expression than spring migrants (Mann–Whitney  $U=158$ ,  $P<0.01$ ; Fig. 1A). There was no significant difference in H-FABP mRNA expression between spring and fall migrants (Mann–Whitney  $U=299$ ,  $P>0.1$ ), and when grouped together H-FABP mRNA expression was 1000% greater during migration than in winter (Mann–Whitney  $U=61$ ,  $P<0.001$ ; Fig. 1A). Within the fall season, there was no effect of age, class or sex on the mRNA expression of any gene investigated (KW  $\chi^2$ ,  $P>0.1$ ). Among all birds, there was no effect of body mass or morph variant (tan or white) on mRNA expression ( $P>0.1$ ).

The expression of FAT/CD36 mRNA and FABPpm mRNA were positively correlated among all birds (Spearman's  $\rho=0.776$ ,  $P<0.001$ ), and within migratory seasons (spring  $\rho=0.568$ ,  $P<0.01$ ; fall  $\rho=0.72$ ,  $P<0.001$ ), but not within wintering birds (winter  $\rho=0.272$ ,  $P>0.1$ ). Furthermore, the expression of H-FABP mRNA was correlated with FAT/CD36 mRNA (Spearman's  $\rho=0.541$ ,  $P<0.001$ ) and FABPpm mRNA (Spearman's  $\rho=0.497$ ,  $P<0.001$ ) among all birds, but when separated by season only fall migrants maintained these correlations (FAT/CD36 Spearman's  $\rho=0.43$ ,  $P=0.008$ ; FABPpm Spearman's  $\rho=0.464$ ,  $P=0.004$ ), whereas spring migrants and wintering birds did not ( $P>0.05$ ).

### Transporter protein variation

All primary antibodies used to detect FAT/CD36 showed non-specific binding and no detectable or quantifiable bands at the

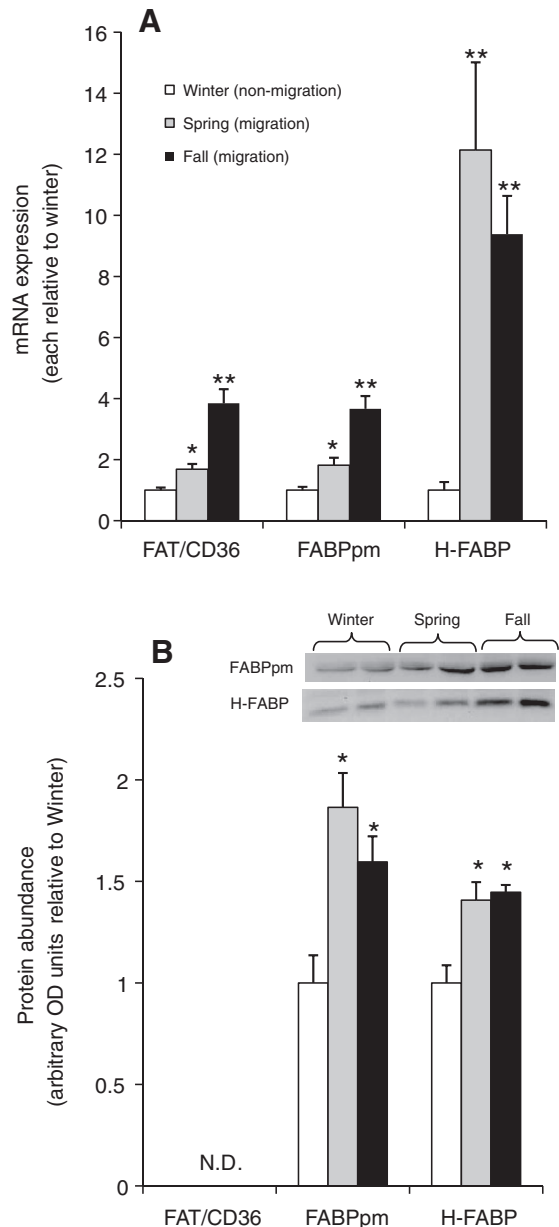


Fig. 1. Expression of FAT/CD36, FABPpm and H-FABP mRNA (A) and protein (B) in pectoralis muscle of white-throated sparrows, separated by season. Expression of each mRNA for each individual bird was compared with a housekeeping gene index (GAPDH and  $\beta$ -actin) and is displayed relative to the mean expression level for wintering birds. \*Significance at  $P<0.05$  level, \*\* $P<0.01$ . N.D., no data.

expected molecular mass. The immunoblot for FABPpm protein showed intense binding of a protein at approximately 40 kDa, which was quantified by optical density relative to a standard run on all gels. The distribution of OD values for FABPpm were skewed (Shapiro–Wilk test for normality,  $P<0.001$ ) and therefore non-parametric tests were used. Overall, there was no effect of body mass, age or sex (KW  $\chi^2$ ,  $P>0.1$ ) on FABPpm protein expression in pectoral muscle. FABPpm protein was not different between spring and fall migrants (Mann–Whitney  $U=195$ ,  $P>0.1$ ). When migrants were pooled together FABPpm was 110% more abundant in pectoral muscle compared with wintering birds (Mann–Whitney  $U=201$ ,  $P<0.001$ ; Fig. 1B). Among all birds the expression of

FABPpm protein was not correlated with its mRNA expression ( $P>0.1$ ). H-FABP protein was 43% more abundant in migratory than in wintering birds ( $P<0.001$ ), but there was no difference between spring and fall migrants ( $P>0.1$ ). There was no correlation between mRNA and protein expression of H-FABP ( $P>0.1$ ).

### Enzyme activities

There was no difference in maximal CS activity between spring and fall birds ( $P>0.1$ ), but when pooled together, activity was increased 90% in migrants compared with wintering birds ( $P<0.001$ ; Fig. 2). Similar 90% and 110% increases in CPT II and HOAD activities were observed, respectively, in migratory birds over wintering birds ( $P<0.001$ ). Compared to wintering birds LDH activity decreased by 30% in fall migrants ( $P<0.001$ ), and further decreased by another 19% in spring migrants ( $P<0.001$ ; Fig. 2). There was no effect of age class, sex, mass or morph on any enzyme activities.

Among migrants only, there was no correlation between any of the enzyme activities and transport protein expression ( $P>0.1$ ). There was, however, a modest correlation between H-FABP mRNA and CPT II activity (Spearman's  $\rho=0.321$ ,  $P=0.028$ ), and CS activity (Spearman's  $\rho=0.383$ ,  $P=0.006$ ). When all birds were pooled together, there were significant correlations between H-FABP protein and enzyme activities of CS (Pearson correlation=0.389,  $P=0.003$ ), CPT II (Pearson correlation=0.349,  $P=0.01$ ) and HOAD (0.416,  $P<0.001$ ), as well as a negative correlation with LDH ( $-0.397$ ,  $P=0.02$ ). FABPpm protein was also correlated with enzyme activities; CS (Spearman's  $\rho=0.429$ ,  $P<0.001$ ), CPT II (Spearman's

$\rho=0.429$ ,  $P<0.001$ ), HOAD (Spearman's  $\rho=0.464$ ,  $P<0.001$ ), but only modestly with LDH (Spearman's  $\rho=-0.238$ ,  $P=0.067$ ).

### DISCUSSION

This is the first study to find that FAT/CD36 (mRNA) and FABPpm (mRNA and protein) are expressed in a non-mammalian species, white-throated sparrows, and furthermore, that the mRNA expression increases in the muscles during migratory seasons. This indicates that migratory white-throated sparrows increase their capacity to take up fatty acids into muscle tissue and can therefore supply the oxidative machinery with a greater influx of exogenous fatty acids. H-FABP expression also increased during migratory seasons. This result is confirmed by two lines of evidence (mRNA and protein) and corroborates previously documented increases in H-FABP protein in migratory western sandpipers (Guglielmo et al., 2002) and barnacle geese (Pellers et al., 1999), confirming our hypothesis that increased capacity to receive FAs at the inside of the plasma membrane, and transport them throughout the cytosol is common among migratory bird species. The seasonal upregulation could also be interpreted as a downregulation during winter seasons when the demand for endurance flight and oxidation of exogenous FA is reduced.

The observation that FAT/CD36 and FABPpm mRNA levels increased by between 70 and 170% during migration supports the hypothesis that enhanced FA uptake across membranes enables endurance flight in birds (Guglielmo et al., 2002; McWilliams et al., 2004). An increase of similar magnitude (90 to 110%) was observed in the maximal activities of oxidative enzymes (CS, CPT II, HOAD) during migration. Other investigations have often detected 15–20% increases in enzyme activity in the flight muscles of birds during migratory seasons (Lundgren and Kiessling, 1985). It is unclear why our results differ from data collected on other species. Fatty acid uptake across muscle membranes is regarded as a possible rate-limiting step in the delivery of exogenous fatty acids from adipose stores to sites of oxidation in muscle mitochondria (Vock et al., 1996). Evidence for this comes from tracer studies showing no increase in exogenous fatty acid oxidation despite increasing available circulating free fatty acid *via* a lipid infusion (Hargreaves et al., 1991; McClelland, 2004). However, with similar magnitudes of change observed in both fatty acid uptake protein abundance and oxidative enzyme capacity, our results indicate that these two could well be matched in white-throated sparrows.

Our evaluation of the data hinges on the assumption that FAT/CD36 plays a role in determining the rate of fatty acid uptake by muscles. In mammalian studies, the abundance of FAT/CD36 and FABPpm on the muscle membrane has been tightly correlated to rates of fatty acid uptake across a range of different muscle fibre types (Koonen et al., 2002), and during muscle contraction (Bonen et al., 2000; Koonen et al., 2004). Overexpression of FAT/CD36 in mammalian muscle is correlated with elevated rates of FA oxidation, but only during muscle contraction, not rest (Ibrahimi et al., 1999). Conversely, FAT/CD36 knockout mice have a normal appearance, but with 50–80% decreased ability for FA uptake into muscle tissue and poor endurance exercise performance (Coburn et al., 2000; Bonen et al., 2007b).

Although we have not measured fatty acid uptake in birds, it is fair to speculate that FAT/CD36 and FABPpm protein abundance on muscle membranes will probably be correlated with the rate of fatty acid uptake. Sweazea and Braun (Sweazea and Braun, 2006) confirmed the presence in sparrows of a protein that functions similarly to mammalian FAT/CD36, by using a known inhibitor (SSO) of FAT/CD36 and observing a 64% decrease in FA uptake in incubated muscles. Although our study did not specifically address

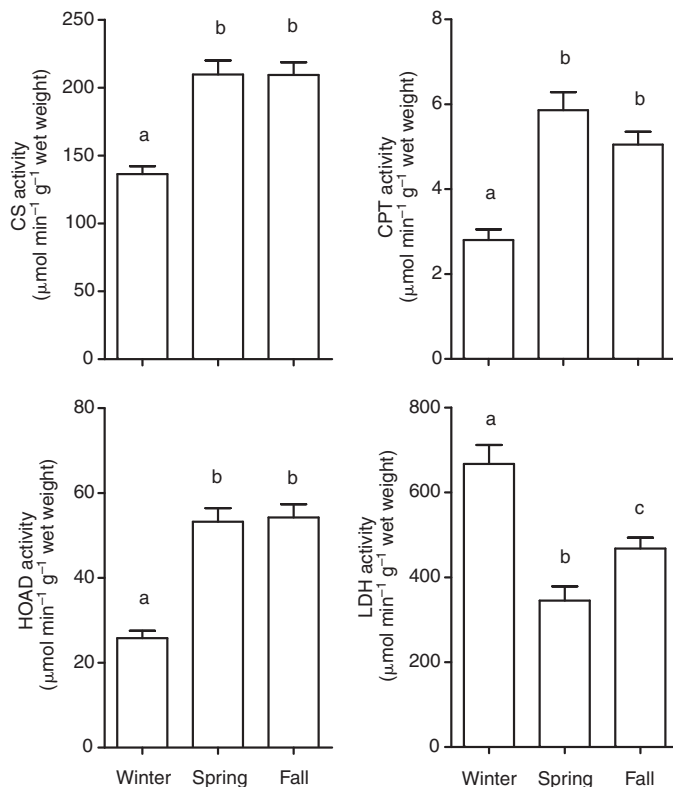


Fig. 2. Maximal enzyme activities of carnitine palmitoyl transferase (CPT), citrate synthase (CS), 3-hydroxyacyl-CoA-dehydrogenase (HOAD) and lactate dehydrogenase (LDH) in pectoralis muscle of white-throated sparrows, separated by season. Different letters above the bars indicate statistical differences. All  $P$ -values  $<0.05$ .

the functional role of FAT/CD36 or FABPpm in birds, the high conservation of the peptide sequences strongly suggests that these proteins serve important functions for individual performance. Furthermore, the observed increase in, (1) the activity of enzymes involved in FA metabolism (CS, CPT II and HOAD) and (2) the abundance of a cytosolic carrier of FA (H-FABP) would probably necessitate an increase in the supply of substrates for oxidation. Therefore, although we were not able to measure FA uptake or FAT/CD36 protein in this study, there is substantial evidence that FA uptake into muscle tissue increases during migratory seasons.

All available antibodies failed to detect any quantifiable band at the predicted molecular mass for FAT/CD36. This is probably attributable to FAT/CD36 being a highly glycosylated, post-transcriptionally modified protein (Hoosdally et al., 2009). Although similar in peptide sequence, the epitopes for which the mammalian-derived antibodies were designed may not be sufficiently similar in white-throated sparrows.

FABPpm mRNA and FABPpm protein levels were not correlated among all birds or within seasons. Since FABPpm protein can be translocated *via* contraction or insulin-induced pathways in mammals (Bonen et al., 2007a; Holloway et al., 2007), the lack of correlation between mRNA and protein in birds might be influenced by the elapsed time since migratory flight or nutritional status. Controlling for activity and diet in a wild population is a difficult task. However, assaying serum triglycerides or  $\beta$ -OH-butyrate levels may provide some clues as to whether or not individual birds were feeding or fasting, respectively (Jenni-Eiermann and Jenni, 1991; Guglielmo et al., 2005). For example, if a particular bird had high serum triglycerides, which would indicate recent feeding, and low  $\beta$ -OH-butyrate, which would indicate no immediately recent migratory activity, we might expect membrane-bound FABPpm protein to be relatively low in abundance.

The expression of FAT/CD36, FABPpm and H-FABP mRNAs was greater during fall migration than spring migration. The reason for this difference is not clear, however, it may indicate different hormonal regulatory systems that control or cue the two migrations. Steroid and thyroid hormones appear to have a strong influence on migratory behaviour during spring and fall, respectively (Butler and Woakes, 1990; Ramenofsky and Wingfield, 2007). Thyroid hormones have long been known to stimulate mitochondrial biogenesis, and more recently have been demonstrated to act *via* peroxisome proliferator-activated receptor  $\gamma$  coactivator 1 $\alpha$  (PGC1 $\alpha$ ) in mammalian muscle (Irrcher et al., 2003). As the name suggests, PGC1 $\alpha$  often acts in concert with peroxisome proliferator-activated receptor  $\gamma$  (PPAR $\gamma$ ), and many genes involved in lipid metabolism have PPAR response elements (PPREs) incorporated to their promoter region. Further investigation of the chicken FAT/CD36 gene (ENSGALG0000008439) revealed a PPRE at -44 before exon 1 (5'-tcaagtgtgtgtt-3'), similar in sequence to a reported PPRE in human and mouse (Teboul et al., 2001). To our knowledge, this is the first report of a possible PPRE upstream of chicken FAT/CD36. The idea of a PPAR-responsive FAT/CD36 in avian muscle seems attractive, considering this possible PPRE and the evidence of differential expression between migratory seasons. The development of new avian-specific antibodies as well as hormone- and photoperiod-manipulation experiments will help elucidate the triggers for the observed seasonal increases.

In summary, the expression of FAT/CD36, FABPpm and H-FABP mRNA are all increased during migratory seasons in the flight muscles white-throated sparrows. Membrane abundance of FABPpm protein and total cellular H-FABP also increase during migration. The results indicate that migratory birds probably increase

FA uptake into flight muscles to power the demanding task of migratory flight by supplying more substrates for oxidation. Results also confirm previous studies of intracellular H-FABP being upregulated during migration. The magnitude of this increase provides evidence for the hypothesis that increased supply of exogenous FA for oxidation is what allows birds to undertake the physiological feat of migratory flight. It also indicates that the abundance of H-FABP mRNA may be a sensitive indicator of migratory state in birds, which we suggest could be extremely useful in future experimental studies of the mechanisms controlling the expression of the migratory state at the muscle biochemical level (e.g. hormones, poly-unsaturated fatty acids, training). H-FABP could be a useful biomarker for avian migration, especially in a partially migrant species or for assessing changes in migratory preparation in response to climate change. Our results suggest that increased protein-mediated FFA uptake capacity may be an evolutionary adaptation to high intensity endurance flight and may be common to all migratory bird species.

#### LIST OF ABBREVIATIONS

CPT	carnitine palmitoyl transferase
CS	citrate synthase
DMSO	dimethyl sulfoxide
FA	fatty acid
FABPpm	plasma membrane fatty acid binding protein
FAT/CD36	fatty acid translocase
GAPDH	glyceraldehyde 3-phosphate dehydrogenase
H-FABP	heart-type fatty acid binding protein
HOAD	3-hydroxyacyl-CoA-dehydrogenase
LDH	lactate dehydrogenase
PMSF	phenylmethylsulphonyl fluoride
PPAR	peroxisome proliferator-activated receptor
PPRE	peroxisome proliferator-activated receptor response element
SSO	sulfo- <i>N</i> -succinimidyl oleate

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