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Seasonal dynamics of flight muscle fatty acid binding protein and catabolic enzymes in a migratory shorebird

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Guglielmo, Christopher G., Norbert H. Haunerland, Peter W. Hochachka, and Tony D. Williams. Seasonal dynamics of flight muscle fatty acid binding protein and catabolic enzymes in a migratory shorebird. *Am J Physiol Regulatory Integrative Comp Physiol* 282: R1405–R1413, 2002; 10.1152/ajpregu.00267.2001.—We developed an ELISA to measure heart-type fatty acid binding protein (H-FABP) in muscles of the western sandpiper (*Calidris mauri*), a long-distance migrant shorebird. H-FABP accounted for almost 11% of cytosolic protein in the heart. Pectoralis H-FABP levels were highest during migration (10%) and declined to 6% in tropically wintering female sandpipers. Premigratory birds increased body fat, but not pectoralis H-FABP, indicating that endurance flight training may be required to stimulate H-FABP expression. Juveniles making their first migration had lower pectoralis H-FABP than adults, further supporting a role for flight training. Aerobic capacity, measured by citrate synthase activity, and fatty acid oxidation capacity, measured by 3-hydroxyacyl-CoA-dehydrogenase and carnitine palmitoyl transferase activities, did not change during premigration but increased during migration by 6, 12, and 13%, respectively. The greater relative induction of H-FABP (+70%) with migration than of catabolic enzymes suggests that elevated H-FABP is related to the enhancement of uptake of fatty acids from the circulation. Citrate synthase, 3-hydroxyacyl-CoA-dehydrogenase, and carnitine palmitoyl transferase were positively correlated within individuals, suggesting coexpression, but enzyme activities were unrelated to H-FABP levels.

endurance exercise; fuel selection; lipid transport; metabolism

THE INSTANTANEOUS COST OF flight is high relative to other forms of locomotion; flying birds expend energy at 10 to 15 times basal metabolic rate (BMR), and the minimum cost of flight may be twice the aerobic limit ($\dot{V}O_{2\,max}$) of similarly sized running mammals (4, 38). In the special case of migratory flight, during which this intensity of exercise is maintained for as long as 50 or even 100 h, energy metabolism is almost completely dominated (85–95%) by the oxidation of exogenous fatty acids (FA) delivered to flight muscles from extramuscular adipose tissue (21, 23, 44). The use of stored fat as a metabolic fuel makes migratory flight possible, yet there currently exists no general mechanistic understanding of how birds achieve the high rates of exogenous FA transport and oxidation required to support such high-intensity endurance exercise.

The most complete information on fuel selection during exercise comes from studies of running mammals (including humans). Generally, the relative contribution of FA oxidation to total fuel demand declines as exercise intensity increases, with the balance of energy derived mainly from carbohydrate oxidation (36). Exogenous FA contribute only a small fraction of the energy needed for exercise of even moderate intensity, and near $\dot{V}O_{2\,max}$ exogenous FA oxidation contributes ~10% of energy demand (43, 45). The rate of utilization of exogenous FA appears to be most limited by transport across the sarcolemma (31, 43). In running mammals, endurance training leads to improved FA utilization, but it is biased toward increased reliance on intramyocyte triaclyglycerol (44, 45). Apparently, a major difference between avian and mammalian fuel metabolism is the enhanced capacity for FA uptake by bird muscles, leading to as much as 20-fold higher rates of exogenous FA oxidation in birds.

The rate of FA uptake by muscles is thought to be determined mostly by processes operating at the level of the myocyte (31). FA can rapidly cross the plasma membrane by simple diffusion, but as much as 80% of FA uptake may take place by a saturable, protein-mediated process (3, 25, 31). Inside the myocyte, the heart-type fatty acid binding protein (H-FABP) binds and transports FA through the cytosol (25, 31). H-FABP greatly increases the aqueous solubility of FA, and H-FABP concentration is correlated positively with muscle FA oxidation capacity (17, 25). Cytosolic FABP may be an important factor that determines the rate of uptake of exogenous FA, causing rapid desorption from the plasma membrane, and efficient translocation to sites of esterification and oxidation (31). Avian muscle cells express an H-FABP similar to that found in other species (1, 16, 33).

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Transitions between migration and nonmigration states offer unique natural manipulations to investigate modulation of FA metabolism in birds. For example, flight muscles generally have higher enzymatic capacity to catabolize FA during migration (26, 27, 29). We previously estimated H-FABP concentration in flight muscles of migrating western sandpipers (*Calidris mauri*) to be near 13% of cytosolic protein, severalfold higher than measured in other vertebrate muscles (16). We suggested that high H-FABP levels could be important for FA uptake by myocytes during endurance flight and that H-FABP levels may vary with seasonally changing demands for exogenous FA. Supporting this hypothesis, flight muscle H-FABP levels in adult barnacle geese (*Branta leucopsis*) were found to be higher during premigratory staging than during chick rearing (33).

Shorebirds undertake some of the longest single flights recorded, sometimes covering >4,000 km and lasting several days (34). Some of the highest fat loads (>50% of total body mass) have been measured in this group (34). The western sandpiper is a small (25 g) long-distance migrant shorebird that breeds in western Alaska and eastern Siberia, and it commonly migrates along the Pacific flyway of North America (47). Western sandpipers wintering in Panama provide an excellent model system to study physiological changes associated with migration. In midwinter, adult and juvenile sandpipers are completely nonmigratory and reach their lowest body masses. During the spring premigratory period, adults molt into breeding plumage, increase body mass, and then migrate north, whereas first-year birds remain in a nonmigratory state (P. D. O'Hara unpublished data). This situation allows tests for migration-related changes in adults, using juveniles as a within-site control.

If H-FABP is indeed important for migratory flight, its level in pectoralis muscle should vary in accordance with changing demands for exogenous FA uptake associated with migration. Enzymes involved in FA catabolism should vary in a similar manner. The present study was carried out to investigate the changes of H-FABP and catabolic enzymes in relation to migration, and to examine correlations among the expression patterns of these proteins, to gain insights into the developmental and metabolic mechanisms that make migratory flight possible.

MATERIALS AND METHODS

Animal sampling. Migrating sandpipers were sampled in 1995 and 1996 during stopover at the Fraser estuary, British Columbia, Canada (49°10′N, 123°05′W). During spring (northward) migration (April 25-May 10) in each year, we collected 15 adults of each sex. In each fall, 15 birds of each sex were collected in July (adults) and August (juveniles). Wintering (nonmigratory) and premigratory sandpipers were sampled at Playa el Agallito, Chitré, Panama (8°N, 79°W). Due to permit restrictions on the total number of birds we could collect, we studied only females in Panama. Fifteen females of each age class were collected during the winter from December 15, 1995 to January 8, 1996, and 15 of each age were taken during the premigration period in March 1996.

We captured sandpipers with mist nets (Avinet, Dryden, NY) under permits from the Canadian Wildlife Service and the Instituto Nacional de Recursos Naturales Renovables (Panama). Animal-handling protocols were approved by the Simon Fraser University Animal Care Committee and conformed with the Canadian Committee for Animal Care guidelines. Birds were anesthetized in the field (16), weighed, and bled from a jugular incision. Blood was collected in heparinized pasteur pipettes (rinsed with 1,000 IU/ml porcine sodium heparin) and centrifuged at 6,000 rpm (2,000 *g*) for 10 min. Plasma was stored at −20°C. A sample (0.3–1 g) from the ventral face of the left pectoralis major adjacent to the sternum was taken. In spring and fall 1995, muscle samples were stored at −20°C for up to 9 mo before transfer to liquid N_2 (−196°C). In Panama and 1996 migration seasons, all samples were immediately frozen and stored in liquid N_2 until analysis. In fall 1998, heart samples were taken from 10 adult migrants and stored at −80°C. Birds were dissected for body composition, and fat content was measured by Soxhlet extraction with petroleum ether (15). Plasma triacylglycerol (controlling for free glycerol) was measured with commercial kits (Sigma, WAKO Diagnostics) (15).

Antiserum production. We purified H-FABP from pectoralis muscle as described previously (16). H-FABP (150 μg) in 200 μl of sterile 0.9% NaCl was homogenized with 200 μl of adjuvant (TiterMax Gold), using two syringes. A New Zealand White rabbit was injected subcutaneously in three locations and boosted 4 wk later with 150 μg FABP. After 2 wk, the antibody titer was assessed by Ouchterlony double immunodiffusion. Antiserum (anti-H-FABP) was collected and stored at −80°C.

Western blotting. SDS-PAGE was carried out as described previously (16). Proteins were transferred overnight to polyvinylidene difluoride memebrane (PVDF) by semidry electroblotting (LKB Novablot; Bjerrum-Shafer-Nielsen buffer, 48 mM Tris, 39 mM glycine, 20% methanol, 0.0375% SDS; 0.6 mA/cm^2 constant current). Blots were rinsed with Tris-buffered saline (TBS; 20 mM Tris, 500 mM NaCl, pH 7.5), blocked 1 h in 5% skim milk powder in TBS, 0.05% Tween 20 (TBS-T), and incubated 1 h in anti-H-FABP 1:8,000 in 5% milk TBS-T. The blot was washed with TBS-T, incubated 1 h with goat-anti-rabbit HRP (GAR-HRP; Jackson Scientific; 1:10,000 in TBS-T), washed three times with TBS-T, and washed once with TBS. Color was developed with diaminobenzidine (DAB; 10.5 mg DAB, 20 ml TBS, 15 μl 30% H_2O_2, 2.2 ml 0.3% $NiCl_2$). Tissues (100 mg) from one sandpiper were homogenized in nine volumes of ice-cold 0.1 M PBS (0.1 M sodium phosphate, 154 mM NaCl, pH 7.4) with a high-speed stainless homogenizer (Virtis-shear Tempest). The homogenate was centrifuged (90,000 *g*, 1 h, 4°C), and protein concentration was measured by Bradford assay (Biorad) using BSA standard. Cytosolic concentration of H-FABP in flight muscle was measured by optical densitometry of SDS gels and Western blots. Lyophilized H-FABP was weighed on a microbalance (±0.1 μg) to make a standard (0.5 μg/μl). Cytosolic proteins (6–16 μg) were separated by SDS-PAGE along with a standard curve of H-FABP (0.5–3 μg). After Coomassie staining, gels were scanned with a flatbed transparency scanner, and band intensities were measured with image-analysis software (Image 1.67, National Institutes of Health, Bethesda, MD). Identical Western blots were prepared, but with less protein to compensate for the greater sensitivity of the immunochemical detection (2- to 4-μg samples; 0.125- to 0.625-μg standards).

Enzyme-linked immunosorbent assay. A noncompetitive ELISA was used to measure H-FABP concentrations in pectoralis and cardiac muscle. With 0.05 M PBS (0.05 M sodium phosphate, 154 mM NaCl, pH 7.4), a clear cytosolic fraction was prepared as described above and diluted (3:997 0.05 M PBS) to a protein concentration of ~20 ng/μl. H-FABP standard (0.5 μg/μl) was diluted to 2.5 ng/μl with 0.05 M PBS. H-FABP appeared to be unstable at concentrations below 1 ng/μl. A 20 mg/ml stock solution of 3,3′,5,5′-tetramethylbenzidine (TMB; Sigma) in dimethylsulfoxide was stored at 4°C in darkness. Assays were carried out in 96-well polystyrene microplates (Corning, Hi Binding, Easywash). Each plate was loaded with purified H-FABP (0–5 ng H-FABP, 3 replicates), six cytosol samples (6 replicates), a control migrant bird measured previously by Western blot (5 replicates), and reagent blanks. The control sample was used to verify assay performance, but samples were not normalized to it. Wells were filled with an appropriate volume of 0.1 M sodium carbonate, pH 9.6 to reach a final volume of 100 μl with the addition of H-FABP standard (0.2–2 μl) or sample (1 μl). Plates were shaken, sealed with mylar tape, and incubated overnight at 4°C. Plates were aspirated, blocked 1 h at room temperature with 100 μl 5% skim milk in 0.05 M PBS-0.1% Tween 20 (PBS-T), reaspirated, and incubated for 2 h with 100 μl of anti-H-FABP 1:5,000 in 2.5% milk PBS-T. Plates were washed four times with 200 μl PBS-T in an automated microplate washer (Biotec), incubated 1 h with 100 μl of GAR-HRP 1:10,000 in PBS-T, and washed six times. Color was developed at room temperature by adding 180 μl of 0.05 mg/ml TMB solution (50 μl TMB stock; 19.95 ml 0.1 M sodium acetate, pH 5.0; 13.4 μl 30% H_2O_2) to each well. Plates were shaken repeatedly, and after 5 min, they were read at 650 nm on a microplate spectrophotometer (Biotec EL 340). Background color development due to nonspecific binding of primary and secondary antibodies was negligible (<10% of samples) and was accounted for in the standard curve. Color development in wells containing bound cytosolic proteins but without antibodies did not differ from background. Recovery of H-FABP from spiked samples was close to 100% at up to three times the expected concentrations of samples. The assay was linear from 0 to 5 ng H-FABP, and intra- and interassay coefficients of variation were 14 and 16.4%, respectively.

Enzyme assays. Samples that had been stored immediately in liquid N_2 (winter, premigration, spring and fall 1996) were assayed for activities of citrate synthase (CS), 3-hydroxyacyl-CoA-dehydrogenase (HOAD), and carnitine palmitoyl transferase (CPT). Pectoralis muscle (100 mg) was minced and combined with nine volumes of ice-cold homogenization buffer (20 mM Na_2HPO_4, 0.5 mM EDTA, 0.2% fatty acid-free BSA, 0.1% Triton X-100, 50 μg/ml aprotinin, 50% glycerol, pH 7.4). This buffer maintains stable enzyme activities during freezing (32). Samples were homogenized on ice at moderate speed with a stainless homogenizer (3 × 10 s, 30-s rest) and then sonicated at a wattage low enough to avoid foaming (3 × 10 s, 30-s rest). Homogenates were stored at −80°C for up to 3 wk. Maximal enzyme activities were measured with a Perkin Elmer UV/Visible spectrophotometer fitted with a water-jacketed cuvette holder maintained at 39°C by a Lauda K-2/R circulating water bath. Assays on crude homogenates were carried out in glass cuvettes with a reaction volume of 1 ml. Assay conditions were: CS (EC 4.1.3.7) 50 mM Tris pH 8.0, 10 μl 1:20 diluted homogenate, 0.5 mM oxaloacetate, 0.15 mM acetyl-CoA, 0.15 mM 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB); CPT (EC 2.3.1.21) 50 mM Tris pH 8.0, 10 μl 1:10 diluted homogenate, 5 mM carnitine (omitted for control), 0.035 mM palmitoyl-CoA, 0.15 mM DTNB; and HOAD (EC 1.1.1.35) 50 mM imidazole pH 7.4, 10 μl 1:10 diluted homogenate, 1 mM EDTA, 0.1 mM aceto-acetyl-CoA, 0.2 mM NADH. Actvities were calculated from ΔA_{412} (ϵ = 13.6) for CS and CPT and from ΔA_{340} (ϵ = 6.22) for HOAD. Control rates for CS and HOAD were negligible. Total protein of homogenates was measured as above.

Data analysis. The distribution of muscle H-FABP concentrations and enzyme activities approached normality in each group and did not require transformation. Pearson correlation analysis was used to test for relationships among H-FABP, CS, HOAD, CPT, body mass, pectoralis fat, and plasma triacylglycerol. ANOVA was used to test for variation among sexes, ages, migratory stages, and years. To control experimentwise error at $\alpha = 0.05$, post hoc multiple comparisons of means were made using the Ryan-Einot-Gabriel-Welsch multiple-range test. Analysis of covariance was used to test for effects of sex and body mass on enzyme activities. A linear contrast was used to compare enzyme activities between all migrant females (Fraser estuary) and all nonmigrant females (Panama). Statistical analyses were carried out using SAS.

RESULTS

The polyclonal antiserum was very specific for avian H-FABP (Fig. 1). In the single bird tested, H-FABP protein level was greatest in flight and cardiac muscle, with less detected in gizzard smooth muscle. H-FABP was not detected in the liver or intestinal mucosa, but

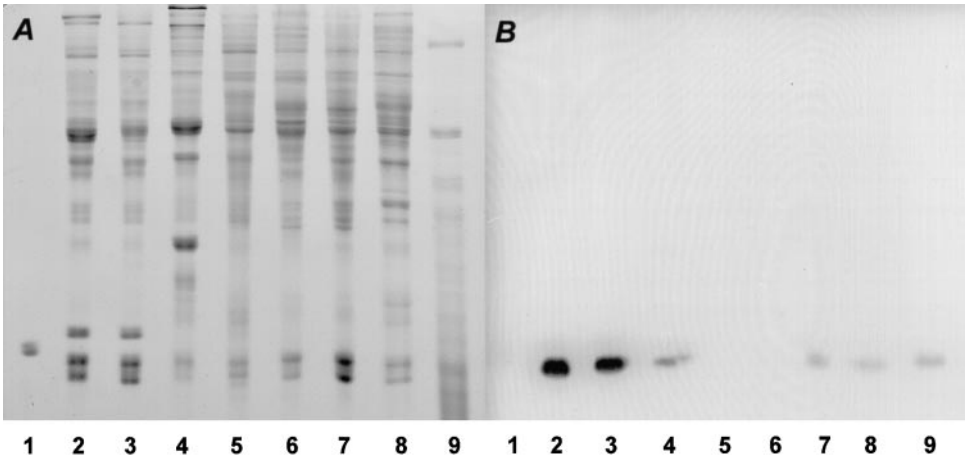

Fig. 1. *A*: SDS polyacrylamide gel of 3 μg desert locust (*Schistocerca gregaria*) heart-type fatty acid binding protein (H-FABP) (*lane 1*) and 10–15 μg of cytosolic proteins from pectoralis muscle (*lane 2*), heart muscle (*lane 3*), gizzard smooth muscle (*lane 4*), liver (*lane 5*), intestinal mucosa (*lane 6*), kidney (*lane 7*), brain (*lane 8*), and pancreas (*lane 9*) of western sandpiper. *B*: Western blot of an identical gel where rabbit-anti-sandpiper-H-FABP antibody was used to detect expression of H-FABP.

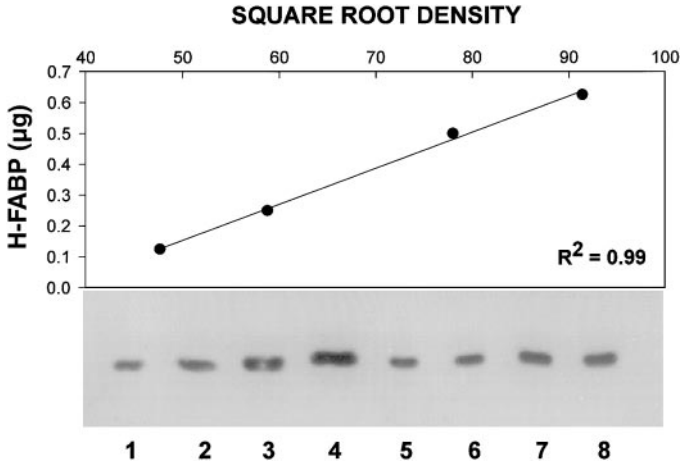

Fig. 2. Western blot used to determine cytosolic concentration of H-FABP in pectoralis muscle of 2 individual migratory western sandpipers. A standard curve was made using 0.125, 0.25, 0.5, and 0.625 μg H-FABP (*lanes 1-4*). Bird samples contained 2 μg (*lanes 5* and *6*) and 4 μg (*lanes 7* and *8*) of cytosolic protein.

the protein (or a similar form) appeared to be present in the kidney, brain, and pancreas. Confirming our previous analysis (16), SDS-PAGE indicated cytosolic concentrations of 13.2 and 13.3% H-FABP in pectoralis muscle of two migrating individuals (not shown). Western blot analysis of the same samples indicated lower concentrations (8.8 and 9.1%; Fig. 2). On the basis of ELISA, H-FABP concentration in cardiac muscle (10.9 ± 0.4%) tended to be slightly higher than in flight muscle of migrants sampled in the same season (fall 1996, 9.5 ± 0.4%; $P = 0.06$).

Within all migrating sandpipers collected in both years, there were significant effects of year and age on flight muscle H-FABP (Table 1). Although not certain, the most likely explanation for the year effect was that storage of samples from spring and fall 1995 at −20°C for several months resulted in poorer tissue preservation and higher calculated cytosolic concentrations of H-FABP (Table 1 and Fig. 3, *A* and *B*). Controlling for the effect of year (i.e., sample storage), fall migrant juveniles had lower flight muscle H-FABP concentrations than adults (Table 1 and Fig. 3, *A* and *B*). Sex was not a significant factor. For seasonal comparisons, data from tissues stored at −20 and −196°C were analyzed separately. H-FABP levels did not vary among winter and premigrant adults and juveniles ($F = 0.4$, df = 3,55, $P = 0.78$) but were 40–66% higher in migrants of both ages in 1996 ($F = 15.5$, df = 6,97, $P = 0.0001$; Fig. 3*B*). H-FABP concentrations did not differ between spring and fall adult migrants in either year of study ($P > 0.72$; Fig. 3, *A* and *B*).

Table 1. *Results of three-factor analysis of variance examining the effects of age, sex, and the year of study on pectoralis muscle H-FABP concentration of western sandpipers sampled during migratory stopover in British Columbia in 1995 and 1996 (n = 173)*

Source	df	Type III SS	*F* Value	Probability
Age	1	32.95	8.74	0.0036
Sex	1	2.02	0.54	0.4653
Year	1	81.53	21.62	0.0001
Age*sex	1	4.78	1.27	0.2618
Age*year	1	0.29	0.08	0.7793
Sex*year	1	10.31	2.73	0.1001
Age*sex*year	1	0.45	0.12	0.7274

H-FABP, heart-type fatty acid binding protein; df, degrees of freedom.

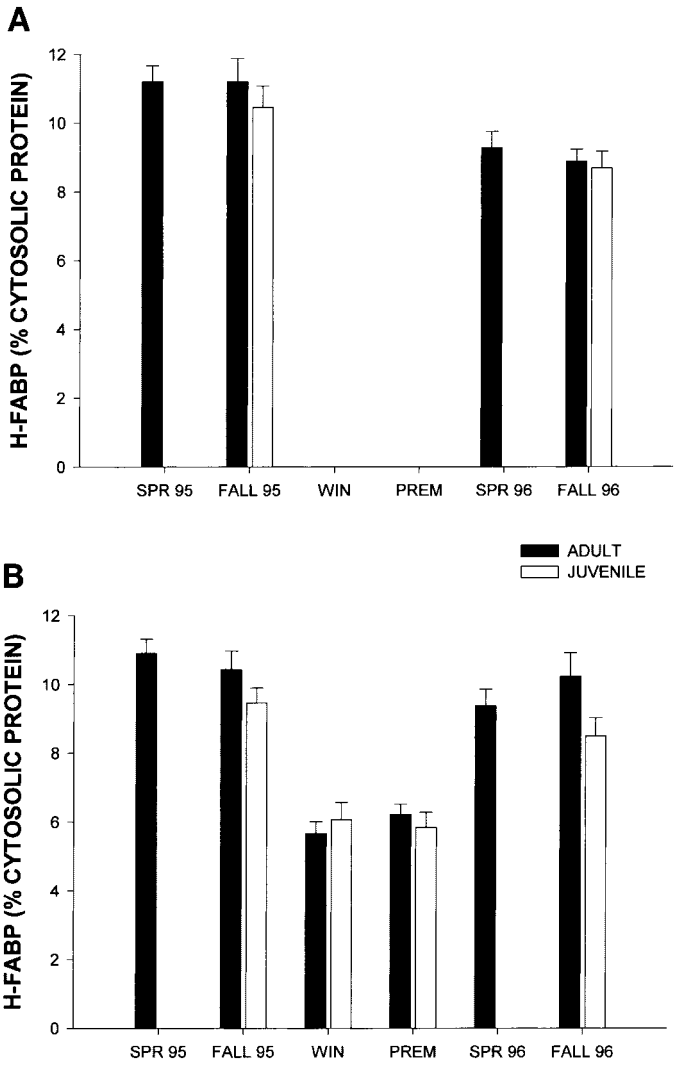

Fig. 3. Seasonal differences in pectoralis muscle H-FABP concentration in adult and juvenile male (*A*) and female (*B*) western sandpipers as determined by ELISA. SPR and FALL indicate spring and fall migrants. WIN and PREM indicate wintering and premigratory sandpipers captured in Panama. See Table 1 and text for statistical analysis.

Catabolic enzyme activities are reported in micromoles per minute per gram wet tissue and in micromoles per minute per milligram protein (Tables 2 and 3). To correct for variation in pectoralis lipid content, only values in micromoles per minute per milligram protein were analyzed statistically. For each enzyme, there was no interaction between body mass and sex ($0.28 > P > 0.88$), and after removing the interaction term, there was no effect of mass with sex in the model

Table 2. *CS, HOAD, and CPT activities in pectoralis muscle of adult and juvenile female western sandpipers*

Season (Site)	Age	*n*	CS	HOAD	CPT
Winter (Panama)	A	15	1.71 ± 0.05*	0.74 ± 0.03*	0.038 ± 0.002*
			356.55 ± 6.22	153.25 ± 4.62	7.84 ± 0.30
	J	15	1.89 ± 0.07*†	0.81 ± 0.04*	0.043 ± 0.002*†
			357.26 ± 7.53	153.46 ± 5.38	8.05 ± 0.36
Premigration (Panama)	A	15	1.87 ± 0.08*†	0.77 ± 0.02*	0.042 ± 0.001*†
			375.73 ± 7.67	153.98 ± 3.67	8.42 ± 0.23
	J	14	1.87 ± 0.06*†	0.76 ± 0.03*	0.038 ± 0.002*
			392.75 ± 8.76	159.50 ± 4.11	8.03 ± 0.30
Spring (Fraser Estuary)	A	15	1.95 ± 0.05*†	0.96 ± 0.04†	0.047 ± 0.002†
			384.29 ± 7.48	190.04 ± 9.58	9.33 ± 0.48
Fall (Fraser Estuary)	A	15	1.89 ± 0.04*†	0.82 ± 0.02*	0.044 ± 0.002*†
			376.37 ± 6.53	162.96 ± 4.26	8.83 ± 0.34
	J	15	2.02 ± 0.06†	0.81 ± 0.02*	0.045 ± 0.001†
			370.42 ± 7.75	149.31 ± 3.95	8.33 ± 0.26

Values are means ± SE [μmol·min^{-1}·mg protein^{-1} (for water and premigration) and μmol·min^{-1}·g wet tissue^{-1} (for spring and fall)]. Within columns, groups not sharing superscripts are significantly different ($P < 0.05$). CS, citrate synthase; HOAD, 3-hydroxyacyl-CoA-dehydrogenase; CPT, carnitine palmitoyl transferase; A, adult; J, juvenile.

($0.18 > P > 0.62$). Removing mass from the model indicated that females had significantly higher CS ($P = 0.03$) and HOAD ($P = 0.0008$) activities than males. A similar trend for CPT was not significant ($P = 0.12$). CS activity varied in females ($F = 2.76$, df = 6,97, $P = 0.02$), but in pairwise comparisons it only differed significantly between wintering adults and fall migrating juveniles (Table 2). CS activity did not differ among samples of migrating males ($F = 0.62$, df = 2,45, $P = 0.58$; Table 3). HOAD activity varied significantly in both sexes (females $F = 5.6$, df = 6,97, $P = 0.0001$; males $F = 4.9$, df = 2,45, $P = 0.01$), and in each sex, HOAD activity was significantly greater during spring migration than all other stages (Tables 2 and 3). CPT activity did not vary among migrating males ($F = 0.4$, df = 2,45, $P = 0.70$; Table 3), but it did in females ($F = 4.2$, df = 6,97, $P = 0.0009$). In pairwise comparisons, spring adults and fall juveniles had higher CPT activity than wintering adults and juveniles in the premigration period (Table 2). Linear contrasts comparing females of all migrant stages to all nonmigrant stages indicated that during migration, CS, HOAD, and CPT activities increased by ~6% ($P = 0.01$), 12% ($P = 0.0001$), and 13% ($P = 0.0001$), respectively.

To avoid spurious correlations arising from coincidental changes in variables with migration, we analyzed migrants (Fraser estuary) and nonmigrants (Panama) separately (Table 4). As might be expected, body mass, percent body fat, and percent pectoralis fat were intercorrelated in migrants and nonmigrants. Plasma triacylglycerol was correlated to body mass and body fat in migrants, but not in nonmigrants, as reported previously (15). Pectoralis H-FABP was weakly correlated to percent pectoralis fat in migrants, but it was unrelated to any of the other variables. In contrast, catabolic enzyme activities were significantly intercorrelated regardless of migratory state. In migrants, CS, HOAD, and CPT were positively correlated with body mass, but this resulted mostly from sex differences in both body size and enzyme activities.

DISCUSSION

Seasonal variation in migratory state naturally alters the requirement for exogenous FA uptake and oxidation by bird flight muscles. In western sandpipers, H-FABP increased from ~6% of cytosolic protein during the winter to ~10% during migration. The greater relative increase of H-FABP concentration (70%) than of HOAD and CPT activities (12–13%) during migration suggests that, rather than simply increasing FA oxidation capacity, high H-FABP is related to acceleration of FA uptake from the circulation. Additional support for this interpretation comes from the observation that captive birds in a nonmigratory state appear to already have the ability to fuel flight by FA oxidation (37, 39), leaving open the question of why migrants increase pectoralis H-FABP concentration if not to alter the path of fuel delivery to favor the use of extramuscular FA.

Table 3. *CS, HOAD, and CPT activities in pectoralis muscle of adult and juvenile male western sandpipers*

Season (Site)	Age	*n*	CS	HOAD	CPT
Spring (Fraser Estuary)	A	16	1.86 ± 0.05*	0.84 ± 0.03*	0.044 ± 0.001*
			371.76 ± 8.73	166.89 ± 4.89	8.78 ± 0.27
Fall (Fraser Estuary)	A	15	1.85 ± 0.05*	0.74 ± 0.02†	0.042 ± 0.002*
			355.98 ± 7.01	142.44 ± 3.59	8.15 ± 0.26
	J	15	1.91 ± 0.05*	0.76 ± 0.02†	0.044 ± 0.002*
			375.13 ± 8.48	149.75 ± 4.10	8.68 ± 0.35

Values are means ± SE [μmol·min^{-1}·mg protein^{-1} (for spring) and μmol·min^{-1}·g wet tissue^{-1} (for fall)]. Within columns, groups not sharing superscripts are significantly different ($P < 0.05$).

Table 4. *Pearson correlation matrix of variables measured in migrating and nonmigrating western sandpipers*

	BM	BF	PF	TRIG	FABP	CS	HOAD
BF	0.79†						
	0.78†						
PF	0.38†	0.57†					
	0.51†	*0.72†*					
TRIG	0.50†	0.40†	0.05				
	0.13	*0.29**	*−0.03*				
H-FABP	−0.05	0.02	0.15*	−0.01			
	0.09	*0.25*	*0.22*	*0.18*			
CS	0.22*	0.17	0.13	0.19	−0.06		
	−0.02	*0.02*	*−0.03*	*0.16*	*0.00*		
HOAD	0.24*	0.10	0.12	0.05	0.19	0.38†	
	−0.01	*0.04*	*0.11*	*−0.10*	*0.06*	*0.77†*	
CPT	0.21*	0.21*	0.24*	0.07	0.01	0.36†	0.50†
	0.17	*0.16*	*0.17*	*0.04*	*0.12*	*0.51†*	*0.63†*

Values are correlation coefficients (r). BM, body mass; BF, % body fat; PF, % pectoralis fat; TRIG, plasma triacylglycerol. For nonmigrants (italics), $n = 59$. For migrants (normal text), $n = 89$ for TRIG, $n = 93$ for enzymes, and $n = 173$ for other variables. *$P < 0.05$, †$P < 0.001$.

Except for HOAD activity, flight muscle biochemistry was similar in spring and fall migrants, as reported for catabolic enzymes in four passerine species (26). Differences in muscle fiber types influenced muscle H-FABP levels in barnacle geese (33). In sandpipers, seasonal variation in biochemistry is unlikely to be caused by shifts in fiber composition as many shorebirds have only a single fiber type in pectoralis (type IIa fast oxidative glycolytic) (11, 12).

Some of the inconsistencies among studies of biochemical modulation in migratory birds may relate to differences in experimental design. When nonmigrants have been compared directly to migrants, flight muscle aerobic capacity (measured by CS, cytochrome oxidase activity, or mitochondrial volume density) has been found to be greater in migrants (11, 26, 27). When birds have been studied over the course of stopover refuelling, aerobic capacity has seemed to be constant or to decrease, even if muscle hypertrophy occurred (8, 11, 29). Marsh's (29) mixed sample of nonmigrant, premigrant, and migrant catbirds (*Dumetella carolinensis*) seems to fit best into the category of stopover studies. In contrast to aerobic capacity, FA oxidation potential (measured by HOAD or CPT) has generally been found to increase on a seasonal basis, as well as during stopover refuelling (this study, 8, 26, 27, 29). In light of these differences, we advocate reservation of the term "premigration" for situations in which birds alter physiology and behavior in anticipation of migration before having yet made any endurance flights.

Premigratory adult sandpipers appeared in their body mass and plumage to be "migration ready" (P. D. O'Hara unpublished data, 15), yet they did not alter pectoralis H-FABP or catabolic enzymes from earlier in the winter. In fact, premigratory adults only deposited fat, without changing lean body composition, liver lipogenic capacity, or adipose tissue fatty acid composition (9, 10, 15). If we compare premigrants to migrants, however, it becomes clear that substantial modulation of all of these physiological characteristics occurred after migratory movements began. These findings imply that before spring migration, western sandpipers may be unable to adjust their physiology in anticipation of future demands. Endurance flight may be required to stimulate expression of H-FABP and catabolic enzymes and to induce hypertrophy of flight muscles and organs such as the heart, even though it would seem adaptive to make these changes in advance of migratory flight. Hyperphagia at stopovers may be required to induce growth of the digestive system and to enhance lipogenic capacity (10, 15). Alternatively, the requirement for "training" may not be an absolute constraint on the induction of physiological systems in sandpipers, but rather it may reflect fitness tradeoffs selecting against maintenance of unneeded capacity during winter and premigration. For example, other tropically wintering shorebirds have depressed BMR associated with decreased size and metabolic activity of organs as a possible adaptation to reduce maintenance energy costs or heat load in a hot climate (22). Under other circumstances, some bird species have been shown to be capable of anticipatory regulation and are not dependent on "use/disuse" mechanisms to modulate their physiology (7, 8, 11, 20). Perhaps anticipatory regulation occurs in situations where a bird must immediately undertake a very long flight with no opportunity for training. Comparative studies of the patterns of physiological change in long- vs. short-hop migrant species could be used to examine the importance of training effects as constraints on the adaptive modulation of physiology.

Age and sex effects. Within 8 to 12 wk of hatching, western sandpiper chicks undertake an overwater flight from western Alaska to British Columbia (5, 47). To maximize survival, these birds are expected to complete development and optimize physiology before departure. Surprisingly, fall juveniles had lower pectoralis H-FABP levels than adults, possibly indicating a reduced capacity to use exogenous FA. Catabolic enzyme activities were similar in adults and juveniles, and other studies show that flight muscles of other young Arctic birds are biochemically mature before migration (2, 24, 33). Nevertheless, low H-FABP levels in juvenile sandpipers and in captive barnacle geese

(33) again suggest that endurance flight training is needed to maximize H-FABP expression.

Human females appear to be better able than males to use FA during exercise (19), and in rats (*Rattus norvegicus*) muscle H-FABP levels are higher in females than males (42). Sex had no effect on pectoralis H-FABP concentration in sandpipers, but catabolic enzyme activities were consistently higher in females. Enzyme differences could be related specifically to sex or to the greater total migration distance of females, which winter farther south than males (47). Sex differences have not been examined in past studies of bird muscle biochemistry, but they should be considered in the future (2, 8, 26, 27, 29).

Tissue-specific H-FABP expression. Similar to other animals, H-FABP expression was greatest in cardiac and skeletal muscle, with small amounts present in gizzard smooth muscle, kidney, and brain, and none detected in liver or intestinal mucosa (14, 41). To our knowledge, H-FABP has not been reported before in the pancreas. Our immunochemical analysis indicates that pectoralis H-FABP concentration is ~30% lower than estimated from SDS-PAGE, but it confirms that H-FABP is a very abundant cytosolic protein (16). Poor tissue preservation at −20°C and the tendency for SDS-PAGE to overestimate H-FABP most likely explain the very high H-FABP concentration in sandpiper cardiac muscle we reported previously (21%) (16).

The concentrations of H-FABP in sandpiper pectoralis and heart are high relative to maximum concentrations found in similar mammalian muscles (2–6%), but they also appear to be greater than measured in two other bird species (1, 13, 17, 31, 33). H-FABP levels in heart, gastrocnemius, and pectoralis were near 5, 2, and 1% of cytosolic protein, respectively, in cold-acclimated, 5- to 6-wk-old ducklings (1). H-FABP levels in barnacle goose pectoralis increased six- to ninefold between the ages of 5 and 12 wk (33), and assuming a similar developmental pattern in ducklings, mature ducks could have pectoralis H-FABP levels comparable to sandpipers. H-FABP concentrations in heart and pectoralis muscles of adult premigratory barnacle geese were 60 and 90 μg/g wet wt, respectively (below 1% of cytosolic protein) (33). Expressed in similar units, sandpiper H-FABP levels were ~6,000 μg/g wet wt in heart and pectoralis of migrants and have been reported to be 10–700 μg/g wet wt in mammalian skeletal muscle, 740–2,600 μg/g wet wt in mammalian heart, and 13,000 μg/g wet wt in flight muscle of desert locusts (13, 17, 18, 33, 48). The very low H-FABP levels in barnacle goose muscles are unexpected and invite further study.

Coregulation of H-FABP and catabolic enzymes. Significant positive correlations among CS, HOAD, and CPT activities indicate that expression of these enzymes may involve similar control elements, especially with respect to FA metabolism. Indeed, genes for both CPT and HOAD have been demonstrated to contain a functional peroxisome proliferator response element (PPRE) that can be activated by long-chain FA (6). We found no correlation between H-FABP expression and activities of any of the enzymes we measured, indicating that the H-FABP promoter is controlled by different factors than these enzymes. Unlike liver FABP, the upstream promoter sequence of the human H-FABP gene does not contain a PPRE, and thus peroxisome proliferator-activated receptors may not be involved in control of H-FABP expression (6, 35, but see 40). Understanding how environmental influences, diet, and activity patterns (training) interact with complex regulatory systems to influence gene expression, metabolic phenotypes, and ultimately animal performance poses an exciting challenge for future research.

Endurance running models for flying birds? Marsh (29) was the first to point out that birds must have "adjustments producing increased oxidation of fatty acids," because in mammals, carbohydrate, not FA, was the fuel limiting the duration of high-intensity exercise. Since that time, it has become even more clear that our understanding of exercise physiology in running mammals is inadequate to describe avian flight. In mammals, the mix of metabolic fuels used during running is a function of relative exercise intensity (e.g., percent $\dot{V}_{O_2\,max}$) rather than absolute work load (36). Up to ~30% $\dot{V}_{O_2\,max}$, fuel demand can be met completely by FA oxidation, but tracer studies indicate that at best only about one-half of this is accounted for by exogenous FA (45). Thus to operate solely on exogenous FA, a running mammal is limited to very low metabolic rates (10–20% $\dot{V}_{O_2\,max}$). This model of fuel selection could apply to birds if energy expenditure in flight represented a low proportion of $\dot{V}_{O_2\,max}$; however, this is not supported by the available data. Allometric scaling equations for $\dot{V}_{O_2}$ flight (2.43 $M_b^{0.72}$; in ml O_2 s^{-1}, M_b in kg) and $\dot{V}_{O_2\,max}$ (2.90 $M_b^{0.65}$) suggest that in the range of 10 g-1 kg, flying birds exercise at 60–85% $\dot{V}_{O_2\,max}$ (38). The majority of ATP production in a mammal running at these intensities would result from carbohydrate oxidation (mostly glycogen), and the FA oxidized would come predominantly from intracellular triacylglycerol (36, 45, 46). In contrast, the avian fuel strategy for migration is based on maximizing the utilization of exogenous FA, with some contribution of protein catabolism for gluconeogenesis and anaplerotic flux (21).

Some of the enhanced utilization of exogenous FA by birds may be explained by microstructural differences between mammalian and avian muscles, but biochemical factors are likely to be very important. The morphometry of capillaries and muscle fibers in mammals appears to be matched to the maximal demand for O_2, and exogenous FA delivery is limited most by sarcolemmal transport capacity (43). Data for the following analysis are very limited, nevertheless as a calculational exercise, it is instructive to compare the dog (*Canis familiaris*) to the rufus hummingbird (*Selasphorus rufus*), a migrant with flight muscles operating close to the theoretical aerobic maximum (39). Hummingbird flight muscles consume O_2 at five times the rate of dog muscles at $\dot{V}_{O_2\,max}$ (33.3 vs. 6.7 $ml \cdot kg^{-1} \cdot s^{-1}$) (39, 43). Capillary surface density is also approximately five times greater in the hummingbird (1,170

vs. 279 cm^{-1}) (30, 43), providing enough additional diffusive capacity to maintain oxygen delivery and most likely a similar mix of fuel substrates (assuming all else equal). Smaller fiber diameter in the hummingbird results in approximately a threefold greater sarcolemmal surface area density (3,343 cm^{-1}; calculated from 30 assuming an average capillary surface-to-fiber surface ratio of 0.35) than in dog muscle (1,158 cm^{-1}) (43). For migratory flight, the hummingbird would require a 10-fold higher FA delivery capacity than the dog, yet it appears that microstructural differences (sarcolemmal surface) alone could meet only one-third of this requirement. Hence, although reduced fiber size may be an important morphological adaptation for migration (28), our analysis highlights why adaptations of the biochemical mechanisms of FA transport may be more critical for the evolution of long-distance migratory flight.

Perspectives

For the bird specialist, it seems obvious that migrants use fat to fuel their extraordinary flights. Yet, for the classically trained exercise physiologist, it seems amazing that adipose tissue can be the major source of fuel for such extremely intense exercise. Our study shows that very high H-FABP levels are probably critical for high rates of FA uptake by bird flight muscles. However, H-FABP is only one component of a complex lipid transport and oxidation system, and a full understanding of the biochemical and physiological mechanisms of endurance flight will require more research. For example, enhanced circulatory delivery of FA by very low density lipoprotein or by albumin with high FA binding capacity has been suggested but not fully explored (21, 44). The existence and function in birds of sarcolemmal transport proteins, such as FA translocase (FAT/CD36), plasma membrane FABP, and FA transport protein, and the role of enzymes such as long-chain acyl-CoA synthetase in intracellular FA processing remain to be studied. Some of these investigations will require novel techniques, such as measuring substrate turnover during endurance flight in a wind tunnel. Other studies can use the natural variability of fuel selection in migrants as a model system. Compared with humans, migratory birds are clearly masters of lipid metabolism, and they promise to teach us much about obesity and how the many components of the lipid transport system work together during exercise.

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