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RESEARCH ARTICLE

Annual life-stage regulation of lipid metabolism and storage and association with PPARs in a migrant species: the gray catbird (*Dumetella carolinensis*)

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ABSTRACT

The annual cycle of a migrating bird involves metabolically distinct stages of substantial fatty acid storage and periods of increased fatty acid mobilization and utilization, and thus requires a great deal of phenotypic flexibility. Specific mechanisms directing stage transitions of lipid metabolism in migrants are largely unknown. This study characterized the role of the PPARs (peroxisome proliferator-activated receptors) in regulating migratory adiposity of the gray catbird (*Dumetella carolinensis*). Catbirds increased adipose storage during spring and autumn migration and showed increased rates of basal lipolysis during migration and tropical overwintering. Expression of the PPAR target genes involved in fat uptake and storage, *FABPpm* and *PLIN3*, increased during pre-migratory fattening. We found significant correlation between PPARγ and target gene expression in adipose but little evidence that PPARα expression levels drive metabolic regulation in liver during the migratory cycle.

KEY WORDS: Avian migration, Passerine, Annual cycle, Peroxisome proliferator-activated receptors, Adipose, Liver

INTRODUCTION

Over the course of a year, birds must cope with a series of seasonal fluctuations in environment and resource availability that drive temporal progression of life-history stages (Wingfield, 2005). Successful transitions from one life-history stage to the next are crucial for fitness but often require drastic adjustments in behavior, morphology and physiology. This need for rapid and substantial changes in a bird's physiology across several stages of the annual cycle makes migrants exemplary models for studying phenotypic flexibility, defined as intra-individual and reversible phenotypic changes (Piersma and Lindstrom, 1997; Wingfield, 2005). However, our understanding of the molecular mechanisms responsible for these phenotypic alterations is limited.

Relative to other stages, migration periods have extremely high energetic cost. Indeed, flapping flight is the most expensive mode of locomotion per unit time (Witter and Cuthill, 1993). Sustained flapping flight over long distances requires substantial fuel stores, most of which are accumulated prior to departure and supplemented with stopover re-fueling in long-distance migrants (Biebach, 1998). Lipids are advantageous as the primary fuel source for migration because fatty acid catabolism yields 8–10 times more energy than glycogen and more metabolic water than both protein and glycogen (Jenni and Jenni-Eiermann, 1998; McWilliams et al., 2004). However, lipids are more difficult to transport through the plasma and cytosol relative to carbohydrates and proteins. Fueling begins with a dramatic increase in food intake, facilitated by hypertrophy of digestive organs and increased digestion efficiency (Bairlein et al., 2013). Diet selection shifts towards foods that promote lipid storage (Bairlein, 2002; Smith et al., 2007).

In mammals, peroxisome proliferator-activated receptors (PPARs) have been identified as key players in lipid metabolism and homeostasis and are likely to play a similar role in avian species (Wang, 2010). Members of this family of nuclear receptor transcription factors are activated by fatty acid ligands and bind to PPAR response elements on DNA as a heterodimer with the retinoid X receptor (RXR). Ligand binding also triggers a conformational change in the receptor that stimulates recruitment of co-activator proteins and loss of co-repressors, ultimately leading to transcription of PPAR target genes (Bensinger and Tontonoz, 2008; Georgiadi and Kersten, 2012).

The three identified isotypes of the PPAR family are functionally differentiated based on tissue distribution and biological function. PPARγ is highly expressed in adipose tissue and drives expression of target genes involved in fatty acid uptake and storage such as lipoprotein lipase and the adipocyte fatty acid binding protein (Bensinger and Tontonoz, 2008). PPARγ can be induced in liver in the context of high dietary fat intake and controls fatty acid synthesis via regulation of genes encoding malic enzyme and fatty acid synthase (Gavrilova et al., 2003; Bedoucha et al., 2001). PPARα is expressed predominately in oxidative tissues such as skeletal muscle and liver. It controls catabolism and utilization of fatty acids through target genes such as adipose triglyceride lipase and fatty acid oxidation pathway genes (Georgiadi and Kersten, 2012). PPARδ (also known as PPARβ) is ubiquitously expressed and activates target genes involved in fatty acid metabolism, mitochondrial respiration and programming of muscle fiber type (Bensinger and Tontonoz, 2008; Wang, 2010; Liu et al., 2013). Here, we focus on two of the three members, PPARγ and PPARα, as the primary regulators of fatty acid storage and utilization.

PPARs are highly conserved in all vertebrates examined thus far, including several avian species. PPARs exhibit a similar tissue distribution in chickens (Meng et al., 2004) and ducks (Wu et al., 2010) to that in mammals. PPARs are also conserved in our study species, the gray catbird (*Dumetella carolinensis*), and show similar molecular function to that in mammals. In functional assays, catbird PPARs respond to fatty acids, isoform-selective ligands, and

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Received 4 April 2016; Accepted 20 August 2016

Journal of Experimental Biology (2016) 219, 3391-3398 doi:10.1242/jeb.141408

List of abbreviations

ATGL	adipose triglyceride lipase
CD36	fatty acid translocase
FABP4	fatty acid binding protein 4
FATP1	fatty acid transport protein 1
HSL	hormone-sensitive lipase
LPL	lipoprotein lipase
PLIN1	perilipin 1
PLIN3	perilipin 3
PPAR	peroxisome proliferator-activated receptor

synthetic antagonists on PPAR-responsive genes, including *LPL* or *CPT1B*, in a pattern comparable to their mammalian homologs (J.M.H., unpublished). However, the involvement of PPARs in seasonal changes in energy uptake, conversion and storage throughout the catbird annual cycle remains unexplored.

The present study aimed to determine the role of PPARs in stage transitions of adiposity across the annual cycle of the gray catbird. Gray catbirds are neotropical migrants that breed throughout north-central North America and overwinter along the Gulf Coast of the USA, the Caribbean and much of Central America. They are abundant in southwest Ohio during spring and summer, and in Belize during the over-wintering months. Several years of banding data in southwest Ohio show that these birds prepare for migration with increased fat stores. Thus, catbirds are an appropriate model species to study physiological changes associated with migration (D.E.R., unpublished observation).

If PPARs are indeed involved in the regulation of adiposity across the annual cycle of a migrant songbird, the expression patterns of PPARs and their target genes should vary in congruence with the morphological and physiological changes observed across life-history stages. We tested the hypothesis that seasonal patterns of adiposity and energy mobilization are associated with coordinated expression patterns of PPARγ and PPARα and their respective target genes. As a primary player in fatty acid uptake and storage, we expected an up-regulation of PPARγ and its targets in adipose and liver during the gray catbird pre-migratory stage. Conversely, we expected elevated expression of PPARα and its target genes involved in fatty acid mobilization and oxidation in the liver during migratory periods. As the liver is the primary lipogenic organ, we also expected it to show mass changes that coordinate with periods of hyperphagia and adipose storage.

MATERIALS AND METHODS

Animal collection

Gray catbirds, *D. carolinensis* (Linnaeus 1766), were captured with mist nets from 30 April to 11 May (late spring migration), 11 to 27 June (breeding), 5 to 15 August (pre-migration) and 17 to 27 September (early autumn migration) in 2013 and 2014, and 8 to 16 January in 2014 (tropical overwintering). Catbirds during spring migration, breeding, pre-migration and autumn migration were caught in southwest Ohio, either at the Hueston Woods Biological Station in Hueston Woods State Park (39°34′ N, 84°44′ W) or at the Miami University Ecology Research Center (39°30′ N, 84°45′ W). Catbirds during tropical overwintering were caught in Indian Church, Belize (17°45′ N, 88°40′ W). All catbirds were caught between sunrise and early afternoon. Upon capture, catbirds were transported to the lab at Miami University (Ohio) or the field laboratory in Indian Church (Belize). All animal trials were approved by the Institutional Animal Care and Use Committee of Miami University (protocol no. 875). Bird capture was permitted through the Ohio Department of Natural Resources, US Fish and Wildlife Services, and the Forest Department of Belize. The experiments complied with the Principles of Animal Care, publication no. 86-23, revised 1985, of the National Institutes of Health as well as the laws of the United States and Belize.

Body composition and tissue collection

Upon capture, all birds were weighed and birds in Ohio were analyzed for whole-body composition using an EchoMRI-SuperFLEX™ analyzer (EchoMRI, Houston, TX, USA). All birds were then killed with an isofluorane overdose and decapitated. The liver was immediately dissected and weighed, and samples of both liver and adipose tissue were placed in Qiagen Allprotect Tissue Reagent for later experiments. Tissues were stored in the Allprotect Tissue Reagent at 4°C for 1–2 days in Ohio, then frozen with liquid nitrogen, and stored at −80°C until further experimentation. In Belize, samples were stored at approximately 4°C for the duration of fieldwork (4–10 days), then frozen at −80°C upon return to the USA.

Rate of basal lipolysis in adipose tissue

An additional 30–200 mg of adipose tissue was removed from each bird and placed in Krebs Ringer buffer (KRB/BSA, made with 15 mmol l^{-1} $NaHCO_3$, 3.32 mmol l^{-1} $CaCl_2$ and 4% fatty acid-free bovine serum albumin; Sigma-Aldrich, St Louis, MO, USA; following Price et al., 2008). Prior to dissections, KRB/BSA was oxygenated with 95% O_2:5% CO_2 for a total of 30 min. Adipose tissue was then minced into ∼1 mm pieces, washed with 50 ml KRB/BSA, and transferred to a 5 ml vial containing 1 ml KRB/BSA warmed to 37°C. The vial was incubated in a 37°C shaking water bath for a total of 2 h and 15 min. Fifteen minutes into incubation, a 100 µl sample of the incubation media was collected. At the end of the incubation, all incubation media was collected. Samples were stored at −20°C until analysis. Media samples from Belize were transported to Ohio for analysis. Samples were analyzed for glycerol concentration as a measure of overall rate of basal lipolysis from the tissue using the Free Glycerol Determination Kit (Sigma-Aldrich) and a spectrophotometer at 540 nm (SpectraMax Plus384, Molecular Devices, Sunnyvale, CA, USA). Glycerol concentration from the first 15 min of incubation were subtracted from the end concentration to correct for any glycerol missed by the initial rinse. The rate of glycerol release is reported as mmol g^{-1} h^{-1}.

Determination of liver lipid content

Liver samples from a subset of animals collected in Ohio were used to determine lipid content. Lipids were extracted from a piece of the liver using a modified Folch method (Folch et al., 1957). Liver samples used in this assay were frozen directly in liquid nitrogen and stored until use. The liver piece was weighed and then minced in 40 ml of 2:1 chloroform:methanol mixture. After 24 h at 4°C, the sample was filtered, 60 ml of 1:1 H_2O:chloroform was added and this was left overnight to separate via gravity in a separatory funnel. The lower phase was drained and evaporated under nitrogen stream using a NEVAP 111 (Organomation, Berlin, MA, USA). The remaining dry extract was weighed and the lipid content (as a percentage of original tissue weight) was obtained.

Expression of PPARs and target genes

RNA was extracted from catbird liver and adipose tissue using TRIzol® Reagent (Ambion, Life Technologies, Carlsbad, CA, USA) according to the manufacturer's instructions and further

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purified with a column cleanup (RNeasy Mini Kit for liver, RNeasy MinElute Cleanup Kit for adipose, Qiagen Inc., Valencia, CA, USA). RNA concentrations and purity were determined using a NanoDrop 2000 (NanoDrop Technologies, Wilmington, DE, USA) and diluted to a final concentration of 0.1 µg µl^{-1} with nuclease-free water. A total of 1 µg RNA was then reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA) following the manufacturer's instructions on a PTC-100 thermocycler (Bio-Rad). cDNA was then used as a template for quantitative real-time PCR (qRT-PCR). Each PCR reaction mixture was composed of cDNA template, 0.1 or 0.05 µmol l^{-1} gene-specific primers (primer-specific optimal concentration) and 2× iQ SYBR® Green Supermix (Bio-Rad) in a total of 15 µl. The temperature cycles for each qRT-PCR reaction were as follows: 3 min at 95°C, 40 cycles of 95°C for 12 s, and a primer-specific optimal temperature (55–63.7°C) for 45 s. Each qRT-PCR run was completed with a melt curve analysis to confirm the presence of a single PCR product and amplification efficiency was verified for every primer pair. Samples from birds collected within each season of the annual cycle were equally represented in each qPCR run for every gene analyzed to ensure variance due to PCR cycle was distributed equally across the experiment. Expression levels were determined using the ΔΔCt method.

We performed RNA-seq analysis of transcripts in adipose collected from two catbirds captured during summer breeding to profile metabolic genes and PPAR isoforms that are expressed in this tissue. The primer sequences corresponding to candidate genes measured in the study were derived from direct sequence data of the annotated data set. Primers were designed to span consecutive exons based on the assumption that the gene organization is conserved between catbirds and the reference organism. Finally, the primers were subject to BLAST analysis to confirm that primers had no other high-affinity recognition sequences in the reference avian genome. Primer sequences are shown in Table 1. Target genes include two long-chain fatty acid membrane transporters (CD36, FATP1), an intracellular fatty acid binding protein (FABP4), two intracellular lipases (HSL, ATGL) and one epithelial lipase (LPL), and two lipid droplet-associated proteins (PLIN1, PLIN3). Transcript expression levels were normalized to 36B4 transcript expression (which did not significantly vary across seasons; $F_{4,32}$=0.642, P=0.636) and are reported relative to the pre-migration stage. The acidic ribosomal protein 36B4, encoded by the *Rplp0* gene, is a commonly used reference gene transcript for normalization because it is ubiquitous and its expression is relatively stable across defined experimental groups (Laborda, 1991).

Table 1. Sequences of primers used for real-time PCR

Gene	Primers
36B4	F: 5′-GCAGACAACGTGGGATCCAAGCAGAT-3′
	R: 5′-GATCCTCCTTAGTGAAGACAAAGCCC-3′
CD36	F: 5′-TAAATGAGTCTGCTGTTATTGG-3′
	R: 5′-GCATATGAAATAGGAGCCCATG-3′
FABP4	F: 5′-TGTGACCATTTTGTGGGCAC-3′
	R: 5′-TTAGGCTTGGCCACACCAGC-3′
FABPpm	F: 5′-TCTGGGGGAAAACAGCGAGGC-3′
	R: 5′-ATGGGAGTGTGATTGCCCC-3′
FATP1	F: 5′-CATCTACACCTCGGGCACCA-3′
	R: 5′-GAGCCGGAACGCGAAATAA-3′
HSL	F: 5′-CTGCGGGAGTACAGCACCAT-3′
	R: 5′-GTAATTCTCGAAGGAGA-3′
ATGL	F: 5′-GTCACCAACGCCAAGAAGGT-3′
	R: 5′-AGTGGGCCCAAGAACCTCTT-3′
LPL	F: 5′-TGCTACCTGGTTCCTGGGCA-3′
	R: 5′-AGCATCCACCAGCTTCGGGA-3′
PLIN1	F: 5′-TTTGCTGTGGCCAACAACCT-3′
	R: 5′-GCTTTTAGCACTTTGGAGGG-3′
PLIN3	F: 5′-TTACGTGCCCATGACGGATG-3′
	R: 5′-ACGAAGTAGCTCTGCTGCTG-3′
PPARα	F: 5′-TGCCACAGCTCCAGGTAGCAT-3′
	R: 5′-CTTGGCCTTCTCAGACCTTGGCATTC-3′
PPARγ	F: 5′-AGCCCTTCACCACCGTTGAC-3′
	R: 5′-AGTTGGACGCCTCCTCGTGA-3′

Statistics

The overall effect of life-history stage on each parameter was analyzed using a one-way ANOVA, followed by *post hoc* comparisons using the Tukey HSD method. If the parametric assumptions of normality and equal variance were not met according to the Shapiro–Wilk and Levene's tests, data were log-transformed. Statistical analyses on the rate of lipolysis and gene expression (unless otherwise noted) were performed on log-transformed data. However, all figures depict untransformed data. In the case of fat mass and lean mass, and expression of *CD36* and *ATGL*, log-transformation still did not meet the one-way ANOVA assumptions, so a non-parametric comparison was used (Wilcoxon and Kruskal–Wallis rank sums test followed by Steel–Dwass pairwise comparisons). A series of linear regressions were calculated to compare relationships between the expression of selected lipolysis or perilipin genes and either the rate of lipolysis or the expression of PPARs. The level of significance was set at P<0.05. All data are reported as means±s.e.m. The number of observations is listed with each figure or table. All statistical procedures were performed with JMP (SAS Institute, version 10.0).

RESULTS

Body mass and composition

Catbirds maintained a significantly higher body mass during early autumn migration while body mass remained relatively constant throughout other stages of the annual cycle (Fig. 1; $F_{4,80}$=9.84, P<0.0001). Fat mass was also elevated during migratory periods compared with breeding and pre-migration, as indicated by MRI

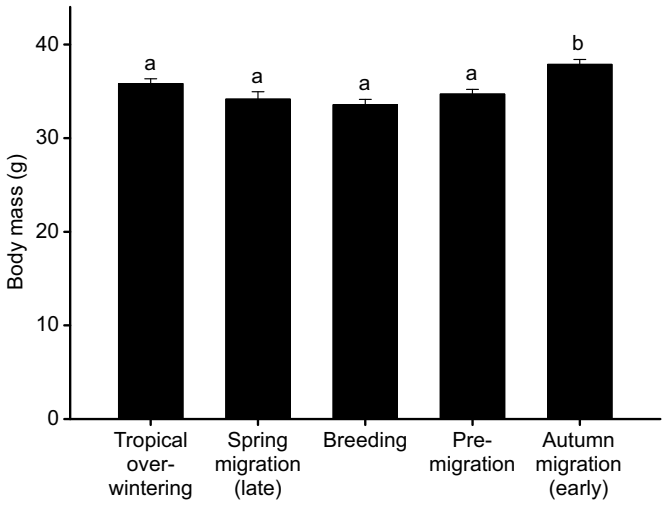

Fig. 1. Body mass of catbirds caught during five stages of the annual cycle. Body mass is highest during autumn migration (N=22) compared with that during tropical overwintering (N=20), spring migration (N=22), breeding (N=14) and pre-migration (N=13). Different letters indicate significant differences following pair-wise comparisons. All data are presented as means+s.e.m.

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Table 2. Seasonal variation in lipid content of catbird livers

	Spring migration (*N*=5)	Breeding (*N*=2)	Pre-migration (*N*=5)	Autumn migration (*N*=4)	*P*
Lipid (%)	8.32±1.67	7.11±0.99	7.46±0.90	6.67±1.11	0.83

measurements (Fig. 2A; χ^2_4=27.10, *P*<0.0001). Lean mass showed significant remodeling across life-history stages (Fig. 2B; χ^2_4=14.57, *P*=0.0022), with the highest lean mass during pre-migration and lowest lean mass measured toward the end of spring migration.

Tissue structure and function

Liver mass from catbirds caught during pre-migration and early autumn migration was significantly greater than that during breeding, late spring migration and tropical overwintering (Fig. 3; $F_{4,48}$=10.55, *P*<0.0001). Lipid content of the livers did not differ across stages (Table 2; $F_{3,12}$=0.29, *P*=0.83). Catbirds caught in the tropics demonstrated the highest rate of basal lipolysis from adipose tissue, compared with the other four life-history stages (Fig. 4; $F_{4,42}$=4.90, *P*=0.0025). Although not different according to *post hoc* comparisons, after grouping migratory or non-migratory catbirds in Ohio only, we found that catbirds mobilized glycerol from adipose tissue at a 1.5-fold higher rate during migratory periods compared with non-migratory periods (*t*-test, *P*=0.005).

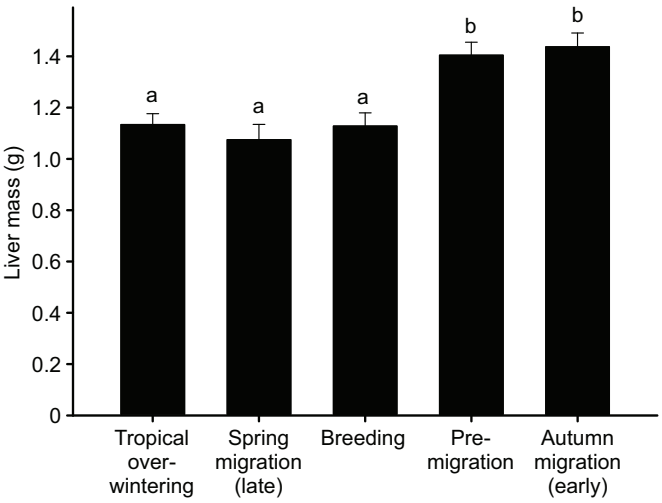

Fig. 3. Liver wet mass of catbirds captured during five stages of the annual cycle. Catbird livers are heavier during periods of prioritized fuel storage (pre-migration, *N*=11; and autumn migration, *N*=10), compared with other stages (tropical overwintering, *N*=10; spring migration, *N*=13; breeding, *N*=8). Different letters indicate significant differences following pair-wise comparisons. All data are presented as means+s.e.m.

PPAR and target gene expression

In the liver, we saw no difference in PPARγ mRNA expression (Fig. 5; $F_{4,61}$=1.07, *P*=0.39). PPARα mRNA expression in the liver did vary across stages of the annual cycle, with the highest levels of expression during spring migration and the lowest expression levels during tropical overwintering (Fig. 5; $F_{4,61}$=5.45, *P*=0.0008). Although PPARα mRNA expression varied, none of the target genes investigated showed a significant change in expression in the liver, although *CD36*, *FABP4* and *LPL* expression did approach significance (Table 3).

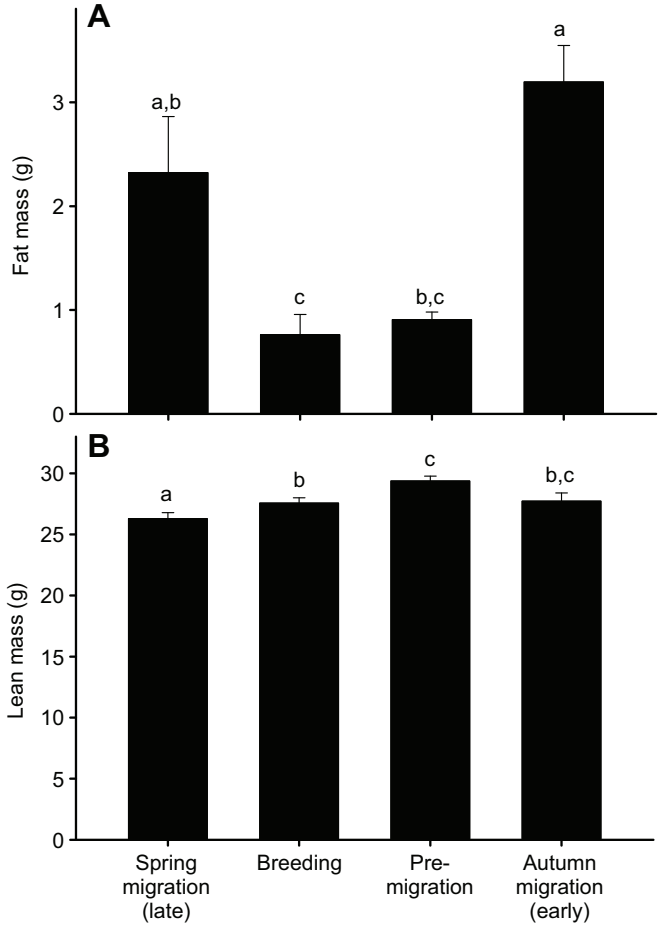

Fig. 2. Body composition of catbirds varies significantly across the annual cycle. (A) Fat mass is highest during spring migration (*N*=22) and autumn migration (*N*=33), compared with that during breeding (*N*=14) and pre-migration (*N*=13). (B) Total lean mass is lowest during spring migration and is higher in the subsequent stages. Different letters indicate significant differences following pair-wise comparisons. All data are presented as means+s.e.m.

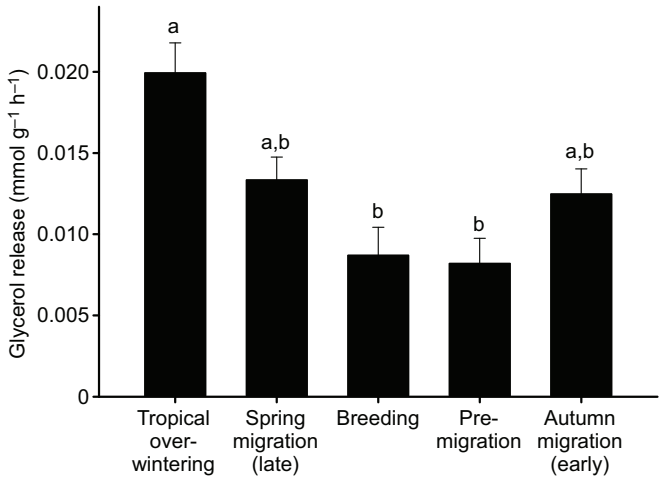

Fig. 4. Basal lipolysis in adipose tissue of catbirds across the annual cycle. The rate of glycerol release (as a measure of basal lipolysis) from catbird adipose tissue is highest during tropical overwintering (*N*=7). Migratory birds exhibit a similarly high rate of basal lipolysis (spring migration, *N*=12; autumn migration, *N*=10), while breeding (*N*=8) and pre-migratory (*N*=10) birds exhibit relatively low rates of basal lipolysis. Different letters indicate significant differences following pair-wise comparisons. All data are presented as means+s.e.m.

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Table 3. Seasonal variation in expression of liver tissue PPAR targets

Gene	Tropical overwintering (*N*=10)	Spring migration (*N*=14)	Breeding (*N*=12)	Pre-migration (*N*=18)	Autumn migration (*N*=12)	*P*
CD36	0.43±0.12	0.47±0.15	0.50±0.17	1.00±0.17	0.59±0.13	0.09
FABP4	0.72±0.33	3.71±1.92	2.36±0.60	1.00±0.23	0.80±0.19	0.11
FABPpm	0.44±0.09	1.18±0.23	0.92±0.19	1.00±0.19	0.79±0.17	0.70
LPL	0.78±0.24	1.55±0.19	1.02±0.19	1.00±0.22	0.53±0.11	0.09
PPARα	0.42±0.08[a]	1.48±0.35[b]	0.89±0.15[a,b]	1.00±0.10[a,b]	0.98±0.21[a,b]	0.0008*
PPARγ	0.65±0.13	1.17±0.29	0.66±0.16	1.00±0.20	1.00±0.20	0.38

Data are presented as means±s.e.m. Asterisks indicate statistical variation across stages. Different superscript letters indicate statistical differences within gene targets, following pairwise comparisons.

In adipose tissue, PPARγ mRNA expression did not vary across stages of the annual cycle (Fig. 5; $F_{4,39}$=0.73, P=0.58). Expression of several of the selected PPAR target genes was not significantly modified across the annual cycle, while *FABPpm* and *PLIN3* expression did vary, and *LPL* expression was very nearly significant (Table 4). *FABPpm* expression in adipose was high during pre-migration and autumn migration, and was significantly depressed during spring migration ($F_{4,39}$=4.85, P=0.003). While *PLIN3* expression did vary across the annual cycle ($F_{4,39}$=2.83, P=0.04), pairwise comparisons did not identify the specific periods that differed. Generally, *PLIN3* expression was highest in the tropics or during pre-migration in Ohio. Using linear regression analysis, a significant relationship was found between expression of *PPARγ* and *ATGL* ($F_{1,42}$=5.23, P=0.027), and between *PPARγ* and *LPL* ($F_{1,42}$=12.65, P=0.0009), both in adipose tissue. However, the r^2 of both was rather low (Table 5). We also found no significant relationship between expression of lipases or perilipins and the rate of glycerol release from adipose tissue, nor between liver and adipose tissue LPL expression (Table 5).

DISCUSSION

The annual cycle of a migratory bird involves several life-history stages, all with distinctly different requirements for lipid metabolism. Each stage entails a unique balance of two prominent physiological states: (1) fuel assimilation and absorption, and (2) fuel mobilization and utilization (Ramenofsky, 1990). Here, we present evidence for the role of PPARs and their target genes in the regulation of fatty acid storage and use across the annual cycle of the gray catbird.

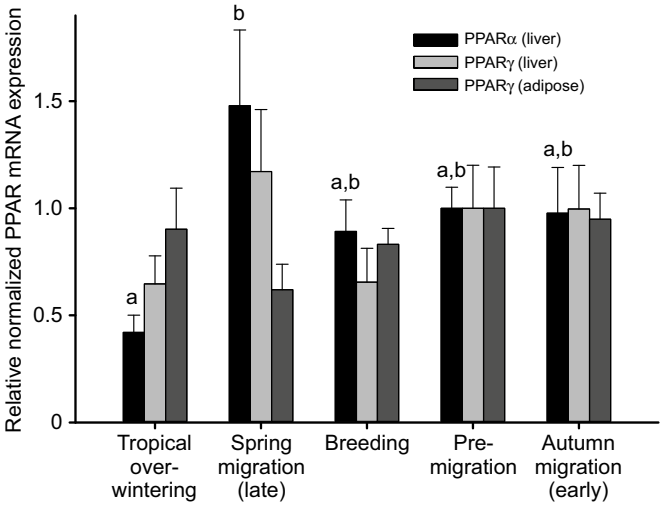

Fig. 5. Seasonal variation in PPAR mRNA expression in the liver and adipose tissues of gray catbirds. mRNA expression of PPARα in the liver varied significantly across stages and was higher during spring migration than during tropical overwintering. PPARγ mRNA expression showed no variation across stages in the liver or adipose tissue. Expression of each transcript was normalized with that of 36B4 and is reported relative to the mean expression for pre-migration birds. Different letters indicate significant differences following pair-wise comparisons. See Tables 3 and 4 for sample sizes. All data are presented as means+s.e.m.

We found increased mRNA expression of PPARα in liver during late spring migration, which suggests a role in fatty acid oxidation during the migratory event. Additionally, these data may support a role for liver PPARα in ketogenesis during prolonged periods without food (i.e. migratory flight), as seen in mammals (Kersten et al., 1999; Leone et al., 1999), to provide adequate energy substrates during periods of fasting. This PPARα mRNA upregulation was not seen during early autumn migration, which is likely a result of differences in sampling between the two migratory periods used in this study. Migratory birds were caught in Ohio at or near the onset of migration (autumn) or near the completion of their northward migration (spring). Lower PPARα mRNA expression during autumn migration suggests a lesser emphasis on lipid oxidation and/or ketogenesis during the onset of migration.

The liver is central to lipid metabolism and plasticity in this organ is expected across the migratory cycle. Elevated liver mass during pre-migration and autumn migration may be attributed to increased lipid accumulation or tissue hypertrophy. Lipogenesis, the synthesis of fatty acids from acetyl CoA in the liver, is carried out by several enzymes including malic enzyme and Δ^9-desaturase – both of which show marked increases in activity during pre-migration (DeGraw, 1975; Shah et al., 1978; Egeler et al., 2000) and are PPAR targets (Yu et al., 2003; Wang et al., 2006). Increased lipogenesis can result in lipid accumulation in the liver, and lipid content is increased in the liver during the pre-migratory period in some species (Odum and Perkinson, 1951; King et al., 1963). However, we found no evidence of increased lipid content within catbird livers despite the elevated mass during pre-migration and autumn migration. Glycogen stores may also contribute to increased liver mass – however, glycogen is a relatively insignificant fuel source for migratory birds so storage is relatively small (Jenni and Jenni-Eiermann, 1998; McWilliams and Karasov, 2001; Guglielmo, 2010). The liver possesses a high capacity for tissue plasticity and regeneration via both hepatocyte hypertrophy and proliferation (Miyaoka et al., 2012). Liver hypertrophy in mammals is typically associated with lipid accumulation and disease (Ludwig et al., 1980). However, increased liver mass is also observed in edible dormice prior to hibernation and is attributed to both lipid accumulation and hypertrophy (Bieber et al., 2011). Thus, we suggest that hepatocyte growth also occurs in response to increased demand for processing of lipid in preparation for migration. We did not see changes in our selected PPAR target genes in liver, which suggests that other, as yet unknown, factors are also important,

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Table 4. Seasonal variation in expression of adipose tissue PPAR targets

Gene	Tropical overwintering (*N*=10)	Spring migration (*N*=7)	Breeding (*N*=5)	Pre-migration (*N*=10)	Autumn migration (*N*=12)	*P*
ATGL	0.92±0.17	0.78±0.19	0.52±0.14	1.00±0.23	0.98±0.20	0.71
FABP4	0.85±0.14	0.89±0.22	0.67±0.09	1.00±0.13	1.21±0.21	0.38
FABPpm	0.74±0.08^{a,b}	0.43±0.05^{b}	0.63±0.15^{a,b}	1.00±0.17^{a}	0.97±0.12^{a}	0.003*
FATP1	0.93±0.30	1.18±0.49	1.44±0.33	1.00±0.18	0.90±0.28	0.83
HSL	1.58±0.85	2.95±1.85	0.57±0.25	1.00±0.22	1.48±0.38	0.62
LPL	0.72±0.17	0.27±0.09	0.59±0.18	1.00±0.26	1.02±0.22	0.05
PLIN1	0.90±0.17	0.60±0.15	0.62±0.17	1.00±0.19	0.89±0.16	0.44
PLIN3	0.92±0.08	0.50±0.09	0.61±0.13	1.00±0.20	0.69±0.12	0.04*
PPARγ	0.90±0.19	0.62±0.12	0.83±0.07	1.00±0.19	0.95±0.12	0.58

Data are presented as means±s.e.m. Asterisks indicate statistical variation across stages. Different superscript letters indicate statistical differences within gene targets, following pairwise comparisons.

although variance in *CD36*/*FAT* expression approached significance, with highest expression levels during pre-migration, when storage is expected to predominate. We did not detect liver hypertrophy in spring migrants, which may be due to measurements being taken at the later stage in migration. Lean mass in general is lower in spring migration, and liver tissue is likely also catabolized both to provide fuel and to reduce the mass of less critical tissues (Bauchinger et al., 2005).

Regardless of whether fatty acids derive from lipogenesis in the liver or from exogenous sources, fatty acid uptake into cells occurs both by passive diffusion and via membrane transporters. Long-chain fatty acids rely solely on facilitated transport across the plasma membrane, involving plasma membrane fatty acid binding protein (FABPpm), fatty acid translocase (CD36/FAT) and fatty acid transport protein (FATP1; Large et al., 2004; Bonen et al., 2007; McFarlan et al., 2009). As pre-migratory birds prefer long-chain unsaturated fatty acids (Pierce et al., 2004), these transport proteins are likely a limiting step for pre-migratory fatty acid storage in adipose tissue. During migration, adipose blood flow is likely important for release of fatty acids as they move down their concentration gradient (Vock et al., 1996). We saw increases in *FABPpm* expression in adipose tissue during pre-migration and into autumn migration in catbirds, indicating a higher capacity for fatty acid uptake and storage into adipocytes. FABPpm in adipose is down-regulated during spring migration, indicating a shift in metabolism away from fatty acid uptake into adipose tissue and likely toward fatty acid transport and utilization in muscle. It may be that fatty acid transporters in adipose are not needed in later stages of migration (e.g. our spring migrating birds) or, alternatively, birds may simply be unable to prioritize their expression after completing most of the migration. Together, these data suggest that increased fat stores during migration are, in part, facilitated by increased expression of fatty acid binding proteins during pre-migration and the onset of migration.

Table 5. Relationship of adipose tissue lipase and perilipin gene expression to the rate of glycerol release, and relationship between target gene and PPAR gene expression

Relationship	*N*	r^2	*P*
ATGL (a) vs glycerol	27	1E−05	0.987
PLIN3 (a) vs glycerol	27	0.003	0.775
HSL (a) vs glycerol	27	0.002	0.843
LPL (a) vs glycerol	27	0.024	0.438
ATGL (a) vs *PPARγ* (a)	44	0.111	0.027*
PLIN3 (a) vs *PPARγ* (a)	44	0.081	0.0620
LPL (a) vs *PPARγ* (a)	44	0.232	0.0009*
HSL (a) vs *PPARγ* (a)	44	0.070	0.083
LPL (l) vs *PPARα* (l)	66	0.019	0.270
LPL (l) vs *LPL* (a)	42	0.0002	0.936

ATGL, adipose triglyceride lipase; *PLIN3*, perilipin 3; *HSL*, hormone-sensitive lipase; *LPL*, lipoprotein lipase. Tissue is denoted as 'a' for adipose or 'l' for liver. Asterisks indicate a significant linear relationship.

Our findings parallel the findings of McFarlan et al. (2009), who found an increase in FABPpm (mRNA and protein) and heart-type fatty acid binding protein (H-FABP) in the muscles of white-throated sparrows (*Zonotrichia albicollis*) during migratory periods. Guglielmo et al. (2002) also found an increase in H-FABP expression in the flight muscles of the western sandpiper (*Calidris mauri*) during migration compared with tropical overwintering and pre-migration. H-FABP, as well as adipocyte fatty acid binding protein (FABP4), mediate fatty acid transport within the cytosol and are in partial control of the metabolic fate of fatty acids within the cell (Bordoni et al., 2006). However, we found no significant seasonal variation in FABP4 expression in either the liver or adipose tissues. The inconsistency of our findings with those of McFarlan et al. (2009) and Guglielmo et al. (2002) regarding intracellular FABP is likely a result of differences in requirements for intracellular fatty acid transport between the tissues measured. Relative to liver and adipose, flight muscles may demand a much more dramatic increase in intracellular fatty acid transport to fuel the aerobic challenge of migration.

To our knowledge, the present study provides the first measure of the rate of basal lipolysis from adipose tissue in wild-caught migrant birds. These data demonstrate a striking increase in energy mobilization during periods of migration, compared with breeding and pre-migratory periods. This result is contrary to a previous report that found no significant variation of mobilization rates with migratory state (Price et al., 2008), although data in that report were nearly significant. Price et al. (2008) used photoperiod manipulations to induce a migratory state in captive white-crowned sparrows (*Zonotrichia leucophrys*), which may not provide an adequate stimulus to elicit a signal for increased energy mobilization, given the lack of activity. Surprisingly, we observed the highest rates of basal lipolysis from catbird adipose tissue during tropical overwintering. These results are perplexing, as catbirds overwintering in the tropics are assumed to have lower energetic demands relative to other life stages. It is also possible that changes in the responsiveness to catecholamines play a role in these patterns.

Mobilization of stored fuels in adipose tissue occurs via lipolysis of tri-acylglycerols. Within adipocytes, hormone-sensitive lipase (HSL) and adipose triglyceride lipase (ATGL) are the major lipases important for controlling lipolysis. ATGL expression is influenced by short-term changes in nutritional status (Kershaw et al., 2006;

Lafontan and Langin, 2009; Serr et al., 2009; Saneyasu et al., 2013a, b). Given the variation in basal lipolysis across the annual cycle, we expected the expression of these two lipases to track seasonal variation. However, we found no differences in expression of either lipase in catbird adipose tissue over the annual cycle, although variation in *ATGL* expression was significantly correlated with PPARγ mRNA expression. In addition to lipases, perilipins are also important in controlling the rate of lipolysis within adipocytes. Perilipins are phosphoproteins that coat lipid droplets in adipocytes to protect triacylglycerol (TAG) against hydrolysis and, in turn, regulate storage of TAG in adipose (Large et al., 2004). Seasonal variation in perilipin expression could thus be relevant to lipid uptake, storage and mobilization. We found no significant variation in perilipin-1 (*PLIN1*) gene expression, but perilipin-3 (*PLIN3*) gene expression was significantly elevated during pre-migratory stages. As PLIN3 is less effective at preventing lipolysis than PLIN1 (Patel et al., 2014), a greater proportion of PLIN3 on the lipid droplets favors lipid mobilization. However, there was no correlation between *PLIN3* expression and lipid release from adipose tissue. Lipoprotein lipase (*LPL*) gene expression varied significantly with PPARγ expression in adipose tissue, but not with lipid export from adipose (Table 5). As adipose LPL is involved in uptake and storage, the latter observation was expected.

This is the first study to address the potential role of PPARs in lipid metabolism of migrant birds across life-history stages. Our data highlight the role of PPARα and PPARγ through coordinated expression changes with their downstream target genes involved in fatty acid uptake and transport during pre-migration and near the end of migration when fat storage and utilization are at their respective maxima. There are many potential PPAR targets in adipose and liver that remain uncharacterized, and future research is necessary to determine whether expression of these genes exhibits seasonal changes.

As regulators of lipid metabolism, PPARs could contribute to the link between environment and phenotype in response to energetic challenges that occur at each life-history stage. Our results show that PPAR expression changes do not vary dramatically (>3-fold) across the annual cycle in liver and adipose, suggesting that PPARs may not be the dominant factors driving metabolic processes. However, as ligand-activated receptors, it is likely that fluctuations in fatty acids (circulating concentrations and species) play a significant role in PPAR-dependent gene reprogramming at various life stages. The diet selection of migratory birds shifts toward fatty acids prior to migration (Bairlein, 2002; McWilliams et al., 2004), but how dietary-derived fatty acids interact with PPARs to regulate downstream target genes is unclear. Additional studies to characterize the seasonal changes in PPAR ligands found in the serum and tissues of birds will be essential to further define the role of PPARs in metabolic regulation during migration. In conclusion, although the lipid metabolism regulatory function of PPARs is conserved across taxa, it is still unclear whether PPARs play a role in regulating changes in lipid metabolism among migratory and non-migratory seasons in birds.

Acknowledgements

We thank Michael Oxendine, Jackie Wagner, Dr Jill Russell and the Avian Research and Education Institute volunteers for assistance with animal capture, and Michael Oxendine and Angela Hamilton for technical assistance. Dr Haifei Shi provided the MRI instrument and Dr Richard Lee provided helpful editorial comments on the manuscript. We also appreciate the assistance of Dr Ann Rypstra and the staff at the Miami University Ecology Research Center. We are grateful for the assistance of Dr Andor Kiss and the staff at the Miami University Center for Bioinformatics and Functional Genomics. We also thank Dr Xiwei Wu and Charles Warden of the City of Hope Functional Genomics Core (supported by the National Cancer Institute of the National Institutes of Health under award number P30CA33572) for performing the RNA-seq annotation.

Competing interests

The authors declare no competing or financial interests.

Author contributions

P.J.S. and J.M.H. conceived and designed the experiments. K.R.C., K.J.D., J.M.H. and P.J.S. participated in the collection of the data. K.R.C. and K.J.D. analyzed the data and performed statistical analysis. K.R.C. wrote the first draft of the manuscript. K.R.C., D.E.R., J.M.H. and P.J.S. revised the manuscript. All authors read and approved the final manuscript.

Funding

This work was funded by National Science Foundation grant IOS-1257455 (to P.J.S., D.E.R. and J.M.H.), a Journal of Experimental Biology (The Company of Biologists) Travelling Fellowship (to K.R.C.), and funds from Miami University (to P.J.S. and K.R.C.).

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