J Exp Biol Advance Online Articles. First posted online on 2 September 2016 as doi:10.1242/jeb.141408
Access the most recent version at http://jeb.biologists.org/lookup/doi/10.1242/jeb.141408

Annual life-stage regulation of lipid metabolism and storage and association with PPARs in the migrant species Gray Catbird (*Dumetella carolinensis*)

Keely R Corder[1,3], Kristen J DeMoranville[1,4], David E Russell[1], Janice M Huss[2], Paul J Schaeffer[1*]

[1]Department of Biology, Miami University, Oxford OH, 45056 USA; [2]Department of Diabetes and Metabolic Diseases Research, Division of Cellular and Molecular Diabetes Research, Beckman Research Institute, City of Hope, Duarte CA, 91010; [3]Current address: Department of Animal and Rangeland Sciences, Oregon State University, Corvallis OR 97331; [4]Current address: Department of Natural Resources Science, University of Rhode Island, Kingston, RI 02881.

Keywords: avian migration, passerine, lipid metabolism, peroxisome proliferator-activated receptors, adipose, liver.

*Address for Correspondence:
Paul J. Schaeffer
Dept. of Biology
212 Pearson Hall
Miami University
Oxford, OH 45056
(513) 529-3624
(513) 529-6900 (fax)
schaefpj@miamioh.edu

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 nuclear receptors, peroxisome proliferator-activated receptors (PPARs), in migratory adiposity of the Gray Catbird (*Dumetella carolinensis)*. Catbirds increased adipose storage during spring and fall 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.

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 requires 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 & 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, much 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 eight to ten times more energy than glycogen and more metabolic water than both protein and glycogen (Jenni & 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). 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 & Tontonoz, 2008; Georgiadi & 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 & 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 & 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, 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 in several avian species. PPARs exhibit a similar tissue distribution in chickens (Meng et al., 2004) and ducks (Wu et al., 2010) as in mammals. PPARs are also conserved in our study species, the Gray Catbird (*Dumetella carolinensis*), and show similar molecular function compared to mammals. In functional assays, catbird PPARs respond to fatty acids, isoform selective ligands, and synthetic antagonists on PPAR-responsive genes, including LPL or CPT1B, in a pattern comparable to their mammalian homologs (J.M. Huss, 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 aims 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 US, 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 Russell, pers. obs.).

If PPARs are indeed involved in the regulation of adiposity across the annual cycle of

a migrant songbird, 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 hypotheses 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 primary lipogenic organ, we also expected to see mass changes in the liver that coordinate with periods of hyperphagia and adipose storage.

MATERIALS AND METHODS

Animal collection

Gray catbirds (*D. carolinensis*) 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), 17 to 27 September (early fall migration) in 2013 and 2014, and 8 to 16 January 2014 (tropical overwintering). Catbirds during spring migration, breeding, pre-migration, and fall 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 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 #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 Institute 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^{TM} analyzer (EchoMRI, Houston, TX, USA). All birds were then euthanized with an isofluorane overdose and decapitated. 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 d 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 d), 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 (made with 15 mM $NaHCO_3$, 3.32 mM $CaCl_2$, and 4% fatty-acid-free bovine serum albumin; Sigma-Aldrich, St. Louis, MO, USA) following Price et al., 2008) (KRB/BSA). Prior to dissections, KRB/BSA was oxygenated with 95%:5% O_2:CO_2 for a total of 30 min. Adipose tissue was then minced into ~1 mm pieces, washed with 50 ml of KRB/BSA, and transferred to a 5 ml vial containing 1 ml of the 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. After 2 h and 15 min of 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 concentrations from the first 15 minutes of incubation were subtracted from the end concentration to correct for any glycerol missed by the initial rinse. Rate of glycerol release is reported as mmol/g/h.

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 as described (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 40ml of 2:1 chloroform:methanol mixture. After 24 hours at 4 °C, the sample was filtered, 60ml of 1:1 H_2O:chloroform were 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 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 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 template for quantitative real-time polymerase chain reaction (qRT-PCR). Each PCR reaction mixture was comprised of cDNA template, 0.1 µM or 0.05 µM gene-specific primers (primer-specific optimal concentration), and 2X iQ SYBR® Green Supermix (Bio-Rad) for 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 sec, and a primer-specific optimal temperature (55-63.7 °C) for 45 sec. Each qRT-PCR run was completed with a melt curve analysis to confirm the presence of a single PCR product and amplification efficiency 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 catbird 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 with the reference gene 36B4 (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 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).

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 variances were not met according to the Shipiro-Wilks and Levene's tests, data were log-transformed. Statistical analyses on 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, CD36, and ATGL, log-transformation still did not meet the one-way ANOVA assumptions, so a nonparametric 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 ± standard error. 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 fall 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 to breeding and pre-migration as indicated by MRI 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 fall migration was significantly greater than 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 to the other four life-history stages. (Fig. 4, $F_{4,42}$=4.90, p=0.0025). Although not different with 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 to non-migratory periods (t-test, p=0.005).

PPAR and target gene expression

In the liver, we saw no difference in PPARγ expression (Fig. 5; $F_{4,61}$=1.07, p=0.39). PPARα 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 varied, none of the target genes investigated varied significantly in the liver, although CD36, FABP4 and LPL did approach significance (Table 3).

In adipose tissue, PPARγ expression did not vary across stages of the annual cycle (Fig. 5; $F_{4,39}$=0.73, p=0.58). Several of the selected PPAR target genes were not significantly modified across the annual cycle, while FABPpm and PLIN3 did vary, and

LPL was very nearly significant (Table 4). FABPpm expression in adipose was high during pre-migration and fall migration, and was significantly depressed during spring migration ($F_{4,39}$=4.85, p=0.003). While PLIN3 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 were rather low (Table 5). We also found no significant relationship between expression of lipases or perilipins with 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.

We found increased 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 upregulation was not seen during early fall 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 (Fall) or near the completion of their northward migration (Spring). Lower PPARα expression during fall 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 fall 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 fall 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 & Jenni-Eiermann, 1998; McWilliams & Karasov 2001; Guglielmo, 2010). The liver possesses a high capacity for tissue plasticity and regeneration via both hepatocye 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, although variance in CD36/FAT 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 weight 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 by both 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). Since 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 fall 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.

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 to 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 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 to breeding and pre-migratory periods. This result is contrary to previous reports that found no

significant variation of mobilization rates with migratory state (Price et al. 2008), although data in that report was nearly significant. Price et al. used photoperiod manipulations to induce a migratory state in captive White-crowned Sparrows (*Zonotrichia leucophrys*) which may not provide adequate stimuli 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, since 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 plays 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 & Langin, 2009; Serr et al., 2009; Saneyasu et al., 2013a; Saneyasu et al., 2014b). 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 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), but perilipin-3 (PLIN3) 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) 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 if expression of these genes exhibit 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.

LIST OF SYMBOLS AND ABBREVIATIONS

PPAR	Peroxisome proliferator-activated receptor
CD36	Fatty acid translocase
FABP4	Fatty acid binding protein 4
FATP1	Fatty acid transport protein 1
HSL	Hormone sensitive lipase
ATGL	Adipose triglyceride lipase
LPL	Lipoprotein lipase
PLIN1	Perilipin 1
PLIN3	Perilipin 3

ACKNOWLEDGEMENTS

We thank Michael Oxendine, Jackie Wagner, Dr. Jill Russell and the AREI 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 to 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 that they have no competing interests.

AUTHOR CONTRIBUTIONS

PJS and JH conceived and designed the experiments. KRC, KJD, JMH and PJS, participated in the collection of the data. KRC and KJD analyzed the data and performed statistical analysis. KRC wrote the first draft of the manuscript. KRC, DER, JMH and PJS revised the manuscript. All authors read and approved the final manuscript.

FUNDING

This work was funded by National Science Foundation grant IOS-1257455 (to PJS, DER and JMH), a Journal of Experimental Biology Traveling Fellowship (to KRC), and funds from Miami University (to PJS and KRC).

REFERENCES

Bairlein, F. (2002). How to get fat: nutritional mechanisms of seasonal fat accumulation in migratory songbirds. *Naturwissenschaften* **89**, 1-10.

Bairlein, F., Dierschke, V., Delingat, J., Eikenaar, C., Maggini, I., Bulte, M., Schmaljohann, H. (2013). Revealing the control of migratory fueling: An integrated approach combining laboratory and field studies in northern wheatears *Oenanthe oenanthe*. *Curr. Zool.* **59**, 381-392.

Bauchinger, U., Wohlmann, A., Biebach, H. (2005). Flexible remodeling of organ size during spring migration of the garden warbler (*Sylvia borin*). *Zoology* **108**, 97-106.

Bedoucha, M., Atzpodien, E., Boelsterli, U. A. (2001) Diabetic KKAy mice exhibit increased hepatic PPARγ1 gene expression and develop hepatic steatosis upon chronic treatment with antidiabetic thiazolidinediones. *J Hepatol*. **35**, 17–23.

Beibach, H. (1998). Phenotypic organ flexibility in Garden Warblers *Sylvia borin* during long-distance migration. *J. Avian Biol.* **29**, 529-535.

Bensinger, S. J., Tontonoz, P. (2008). Integration of metabolism and inflammation by lipid-activated nuclear receptors. *Nature* **454**, 470-477.

Bieber, C., Außerlechner, K., Skerget, C., Walzer, C., Ruf, T. (2011). Seasonal changes in liver size in edible dormice (*Glis glis*): non-invasive measurements using ultrasonography. *Eur. J. Wildl. Res.* **57**, 657-662.

Bonen, A., Chabowski, A., Luiken, J. J. F. P., Glatz, J. F. C. (2007). Mechanisms and regulations of protein-mediated cellular fatty acid uptake: molecular, biochemical, and physiological evidence. *Physiology* **22**, 15-28.

Bordoni, M., Di Nunzio, M., Danesi, F., Biagi, P. L. (2006). Polyunsaturated fatty acids: from diet to binding to PPARs and other nuclear receptors. *Genes Nutr.* **1**, 95-106.

DeGraw, W. A. (1975). Seasonal changes in liver malic enzyme activity in white-crowned sparrows, *Zonotrichia leucophrys gambelli*. *Proc. Nebr. Acad. Sci. Affil. Soc. 85th Annu Meet*, 12.

Egeler, O., Williams, T. D., Guglielmo, C. G. (2000). Modulation of lipogenic enzymes, fatty acid synthase and delta9-desaturase, in relation to migration in the western sandpiper (*Calidris mauri*). *J. Comp. Physiol. B* **170**, 168-74.

Folch, J., Lees, M., Sloane, G. H. (1957). A simple method for the isolation and purification of total lipids from animal tissues. *J. Biol. Chem.* **226**, 497-509.

Gavrilova, O., Haluzik, M., Matsusue, K., Cutson, J. J., Johnson, L., Dietz, K. R., Nicol, C. J., Vinson, C., Gonzalez, F. J., Reitman, M. L. (2003) Liver peroxisome proliferator-activated receptor γ contributes to hepatic steatosis, triglyceride clearance, and regulation of body fat mass. *J. Biol. Chem.* **278**, 34268-34276.

Georgiadi, A., Kersten, S. (2012). Mechanisms of gene regulation by fatty acids. *Adv. Nutr.* **3**, 127-134.

Guglielmo, C. G., Haunerland, N. H., Hochachka, P. W., Williams, T. D. (2002). Seasonal dynamics of flight muscle fatty acid binding protein and catabolic enzymes in a long-distance migrant shorebird. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **282**, R1405-R1413.

Guglielmo, C. G. (2010). Move that fatty acid: fuel selection and transport in migratory birds and bats. *Integr. Comp. Biol.* **50**, 336-345.

Jenni, L., Jenni-Eiermann, S. (1998). Fuel supply and metabolic constraints in migrating birds. *J. Avian Biol.* **29**, 521-528.

Kershaw, E. E., Hamm, J. K., Verhagen, L. A. W., Peroni, O., Katic, M., Flier, J. S. (2006). Adipose Triglyceride Lipase: function, regulation by insulin, and comparison with adiponutrin. *Diabetes*. **55**, 148-157.

Kersten, S., Seydoux, J., Peters, J.M., Gonzalex, F.J., Desvergne, B., Wahli, W. (1999). Peroxisome proliferator-activated receptor α mediates the adaptive response to fasting. *J. Clin. Invest.* **103**, 1489-1489.

King, J. R., Barker, S., Farnder, D. S. (1963). A comparison of energy reserves during autumnal and vernal migratory periods in the white-crowned sparrow, *Zonotrichia leucophrys gambelii*. *Ecology* **44**, 513-521.

Laborda, J. (1991). 36B4 cDNA used as an estradiol-independent mRNA control is the cDNA for human acidic ribosomal phosphoprotein P0. *Nucleic Acids Research* **19**, 3998.

Lafontan, M., Langin, D. (2009). Lipolysis and lipid mobilization in human adipose tissue. *Prog. Lipid Reg.* **48**, 275-297.

Large, V., Peroni, O., Letexier, D., Ray, H., Beylot, M. (2004). Metabolism of lipids in human white adipocyte. *Diabetes Metab.* **30**, 294-309.

Liu, D., Brown, J. D., Stanya, K. J., Homan, E., Leidl, M., Inouye, K., Bhargava, P., Gangl, M. R., Dai, L., Hatano, B., Hotamisligil, G. S., Saghatelian, A., Plutzky, J., Lee, C.-H. (2013) A diurnal serum lipid integrates hepatic lipogenesis and peripheral fatty acid use. *Nature* **502**, 550-554.

Leone, T.C., Weinheimer, C.J., Kelly, D.P. (1999) A critical role for the peroxisome proliferator-activated receptor α (PPARα) in the cellular fasting response: The PPARα-null mouse as a model of fatty acid oxidation disorders. *Proc. Natl. Acad. Sci. USA* **96**, 7473-7478.

Ludwig, J., Viggiano, T. R., McGill, D. B., Oh, B. J. (1980). Nonalcoholic steatohepatitis: Mayo Clinic experiences with a hitherto unnamed disease. *Mayo Clin. Proc.* **55**, 434-438.

McFarlan, J. T., Bonen, A., Guglielmo, C. G. (2009). Seasonal upregulation of fatty acid transporters in flight muscles of migratory white-throated sparrows (*Zonotrichia albicollis*). *J. Exp. Biol.* **212**, 2934-2940.

McWilliams, S. R.; Karasov, W. H. (2001). Phenotypic flexibility in digestive system structure and function in migratory birds and its ecological significance. *J. Comp. Physiol. A* **128**, 579-593.

McWilliams, S. R., Guglielmo, C., Pierce, B., Klaassen, M. (2004). Flying, fasting, and feeding in birds during migration: a nutritional and physiological ecology perspective. *J. Avian Biol.* **35**, 377-393.

Meng, H., Li, H., Zhao, J. G., Gu, Z. L. (2004). Differential expression of peroxisome proliferator-activated receptors alpha and gamma gene in various chicken tissues. *Domest. Anim. Endocrin.* **28**, 105-110.

Miyaoka, Y., Ebato, K., Kato, H., Arakawa, S., Shimizu, S., Miyajima, A. (2012). Hypertrophy and unconventional cell division of hepatocytes underlie liver regeneration. *Curr. Biol.* **22**, 1166-1175.

Nagahuedi, S., Popesku, J. T., Trudeau, V. L., Weber, J. M. (2009). Mimicking the natural doping of migrant sandpipers in sedentary quails: effects of dietary n-3 fatty acids on muscle membranes and PPAR expression. *J. Exp. Biol.* **212**, 1106-1114.

Odum, E. P., Perkinson, J. D. Jr. (1951). Relation of lipid metabolism to migration in birds: seasonal variation in body lipids of the migratory white-throated sparrow. *Physiol. Zool.* **24**, 216-230.

Patel, S., Yang, W., Kozusko, K., Saudek, V., Savage, D. B. (2014). Perilipins 2 and 3 lack a carboxy-terminal domain present in perilipin 1 involved in sequestering ABHD5 and suppressing basal lipolysis. *Proc. Natl. Acad. Sci. USA* **111**, 9163-9168.

Pierce, B. J., McWilliams, S. R., Place, A. R., Huguenin, M. A. (2004). Diet preferences for specific fatty acids and their effect on composition of fat reserves in migratory Red-eyed Vireos (*Vireo olivaceous*). *Comp. Biochem. Physiol. A* **138**, 503-514.

Piersma, T., Lindstrom, A. (1997). Rapid reversible changes in organ size as a component of adaptive behavior. *Trends Ecol. Evol.* **12**, 134-138.

Price, E. R., Krokfors, A., Guglielmo, C. G. (2008). Selective mobilization of fatty acids from adipose tissue in migratory birds. *J. Exp. Biol.* **211**, 29-34.

Ramenofsky, M. (1990). Fat storage and fat metabolism in relation to migration. In *Bird migration: physiology and ecophysiology* (ed. E. Gwinner). pp. 214-231. Berlin, Germany, Springer-Verlag.

Saneyasu, T., Shiragaki, M., Nakanishi, K., Kamisoyama, H., Honda, K. (2013a). Effects of short term fasting on the expression of genes involved in lipid metabolism in chicks. *J. Comp. Physiol. B* **165**, 114-118.

Saneyasu, T., Shiragaki, M., Nakanishi, K., Kamisoyama, H., Honda, K. (2013b). Effects of short-term refeeding on the expression of genes involved in lipid metabolism in chicks (*Gallus gallus*). *J. Comp. Physiol. B* **166**, 1-6.

Serr, J., Suh, Y., Lee, K. (2009). Regulation of adipose triglyceride lipase by fasting and refeeding in avian species. *Poultry Sci.* **88**, 2595-2591.

Shah, R. V., Patel, S. T., Pilo, B. (1978). Glucose-6-phosphate dehydrogenase and 'malic' enzyme activities during adaptive hyperlipogenesis in migratory starling (*Sturnus roseus*) and white-wagtail (*Motacilla alba*). *Can. J. Zool.* **56**, 2083-2087.

Smith, S.B., McWilliams, S.R., Guglielmo, C.G. (2007). Effect of diet composition on plasma metabolite profiles in a migratory songbird. *Condor*. **109**, 48-58.

Vock, R., Weibel, E.R., Hoppeler, H., Ordway, G., Weber, J.-M., Taylor, C.R. (1996). Design of the oxygen substrate pathways. V. Structural basis of vascular substrate supply to muscle cells. *J. Exp. Biol.* **199**, 1675-1688.

Wang, Y. X. (2010). PPARs: diverse regulators in energy metabolism and metabolic diseases. *Cell Res.* **20**, 124-137.

Wang, Y., Botolin, D., Xu, J., Christian, B., Mitchel, E., Jayaprakasam, B., Nair, M., Peters, J. M., Busik, J., Olson, L. K., Jump, D. B. (2006). Regulation of hepatic fatty acid elongase and desaturase expression in diabetes and obesity. *J. Lipid Res.* **47**, 2028-2041.

Wingfield, J. C. (2005). Flexibility in annual cycles of birds: implications for endocrine control mechanisms. *J. Ornithol.* **146**, 291-304.

Witter, M. S., Cuthill, I. C. (1993). The ecological costs of avian fat storage. *Philos. Trans. R. Soc. Lond. B. Biol. Sci.* **340**, 273-92.

Wu, Y., Liu, X., Xiao, H., Zhang, H. (2010). The differential expression of peroxisome proliferators-activated receptors in various duck tissues. *Mol. Biol. Rep.* **37**, 1235-1240.

Yu, S., Matsusue, K., Kashireddy, P., Cao, W. Q., Yeldandi, V., Yeldandi, A. V., Rao, M. S., Gonzalez, F. J., Reddy, J. K. (2003). Adipocyte-specific gene expression and adipogenic steatosis in mouse liver due to peroxisome proliferator-activated receptor gamma1 (PPARgamma1) overexpression. *J. Biol. Chem.* **278**, 498-505.

Figures

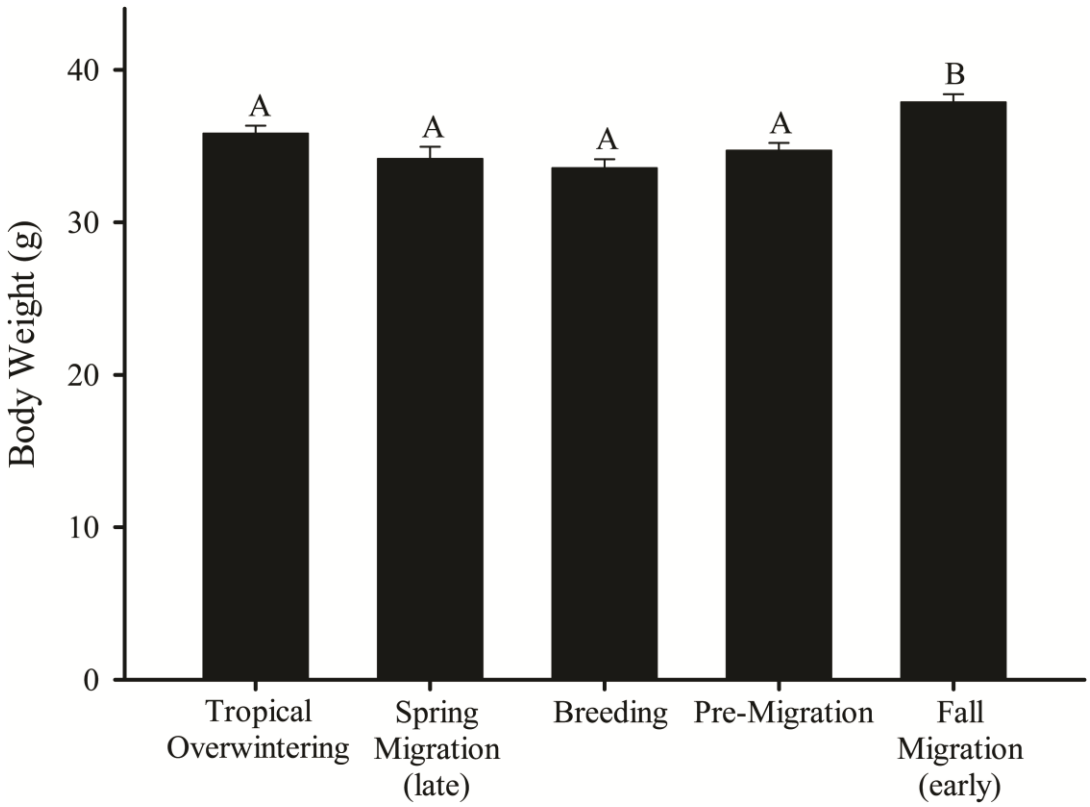

Figure 1.

Catbird body mass caught during five stages of the annual cycle. Body weight is highest during fall migration (n = 22) compared to 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 mean ± SE.

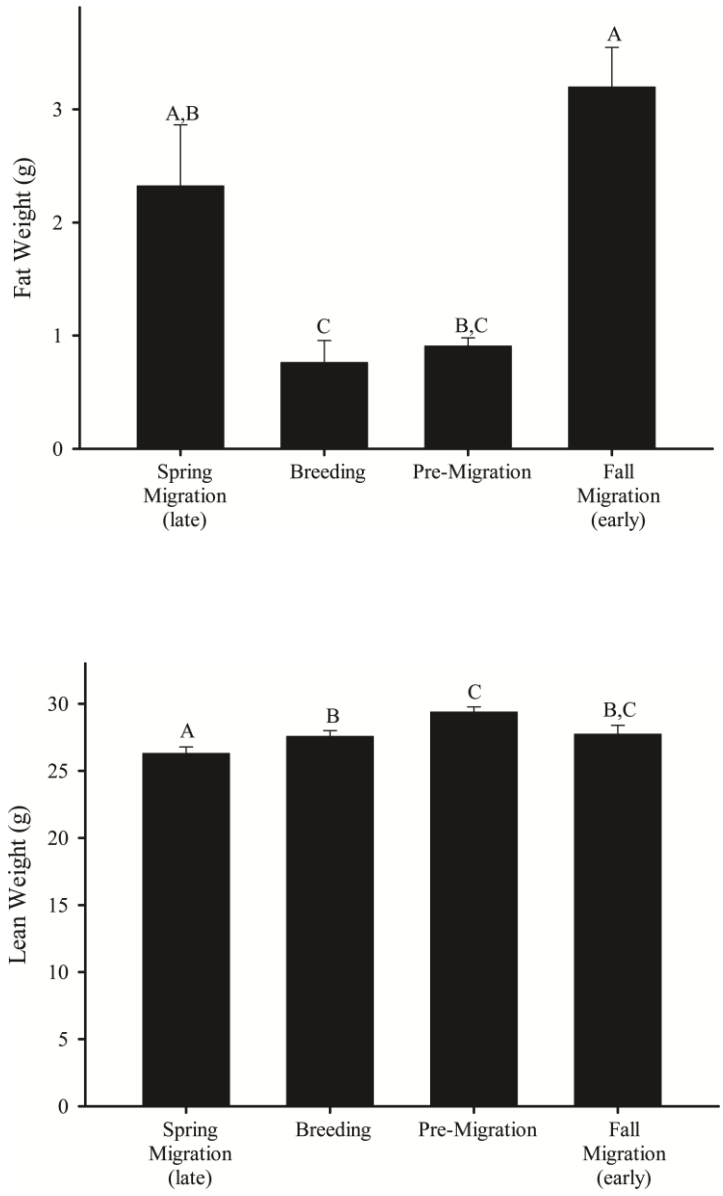

Figure 2.

Body composition of catbirds varies significantly across the annual cycle. Fat mass (A) is highest during spring migration (n=22) and fall migration (n=33), compared to breeding (n = 14) and pre-migration (n = 13). Total lean mass (B) is lowest during spring migration and increases in the subsequent stages (breeding and pre-migration). Different letters indicate significant differences following pair-wise comparisons. All data are presented as mean ± SE.

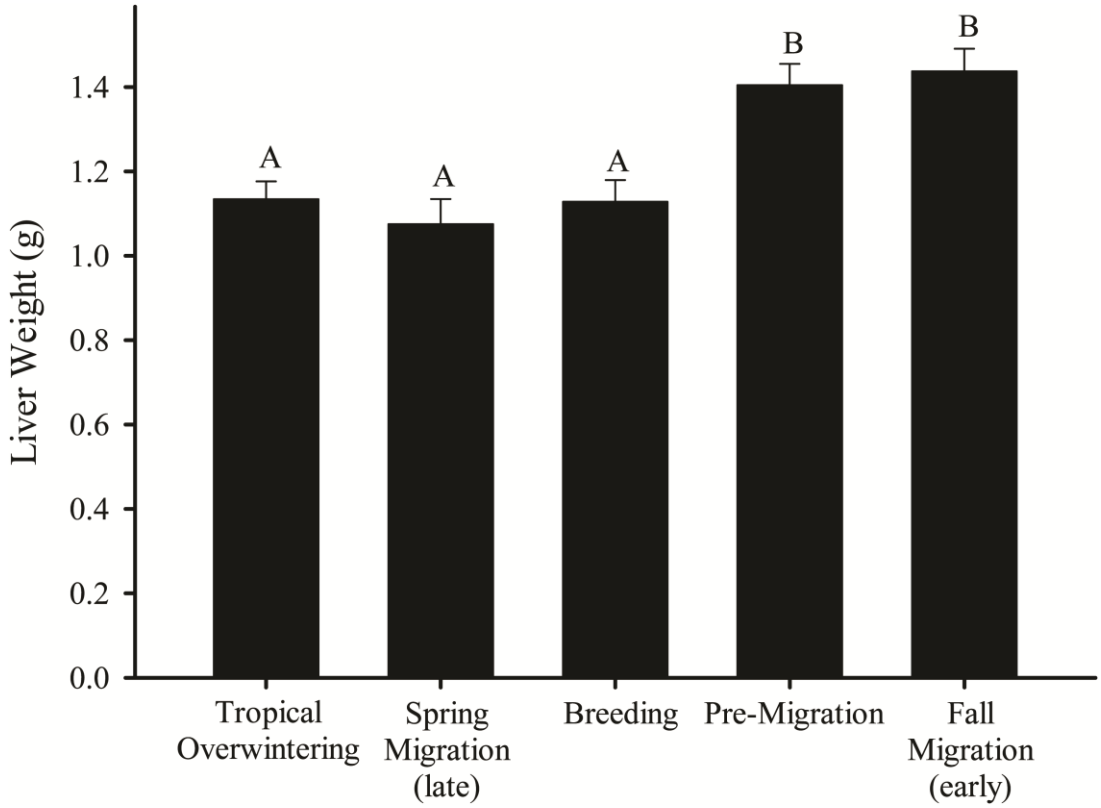

Figure 3.

Wet mass of catbird livers captured during five stages of the annual cycle. Catbird livers were heavier during periods of prioritized fuel storage (pre-migration and fall migration), compared to other stages (tropical overwintering, n=10; spring migration, n=13; breeding, n=8; pre-migration, n=11; fall migration, n=10). Different letters indicate significant differences following pair-wise comparisons. All data are presented as mean ± SE.

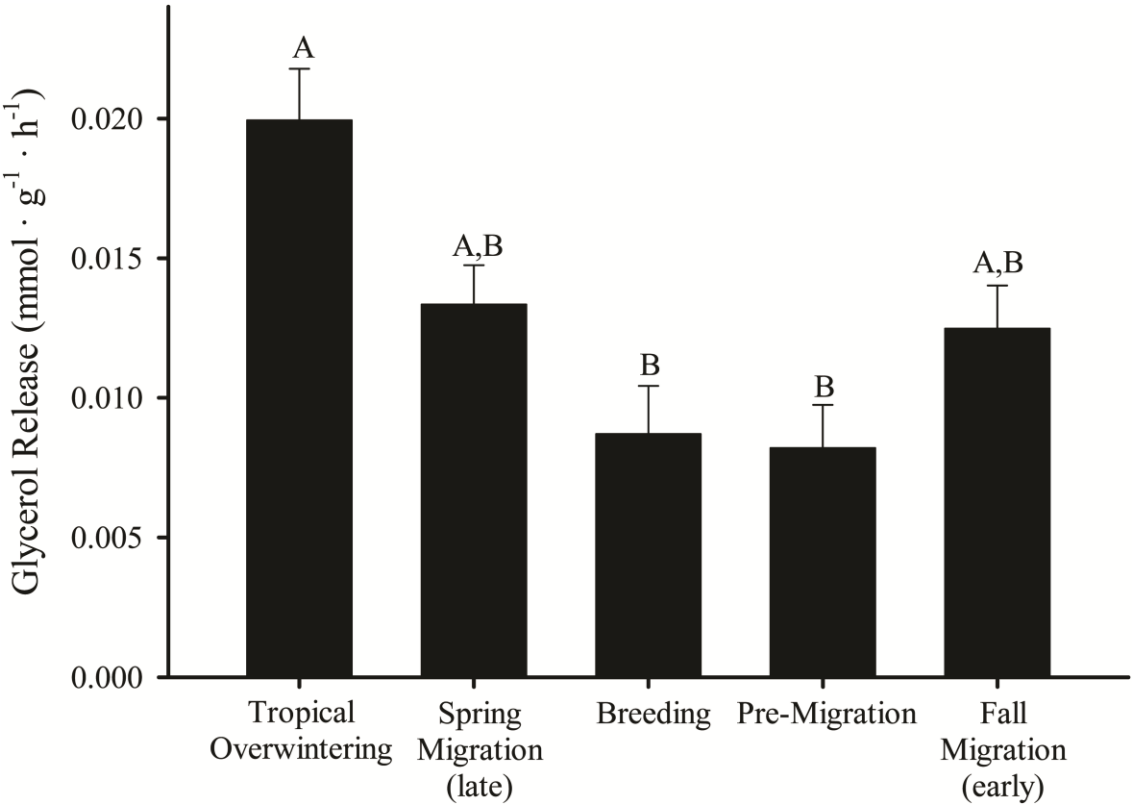

Figure 4.

Rate of glycerol release 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; fall migration, n=10), while breeding and pre-migratory birds exhibit relatively low rates of basal lipolysis (n=8, n=10, respectively). Different letters indicate significant differences following pair-wise comparisons. All data are presented as mean ± SE.

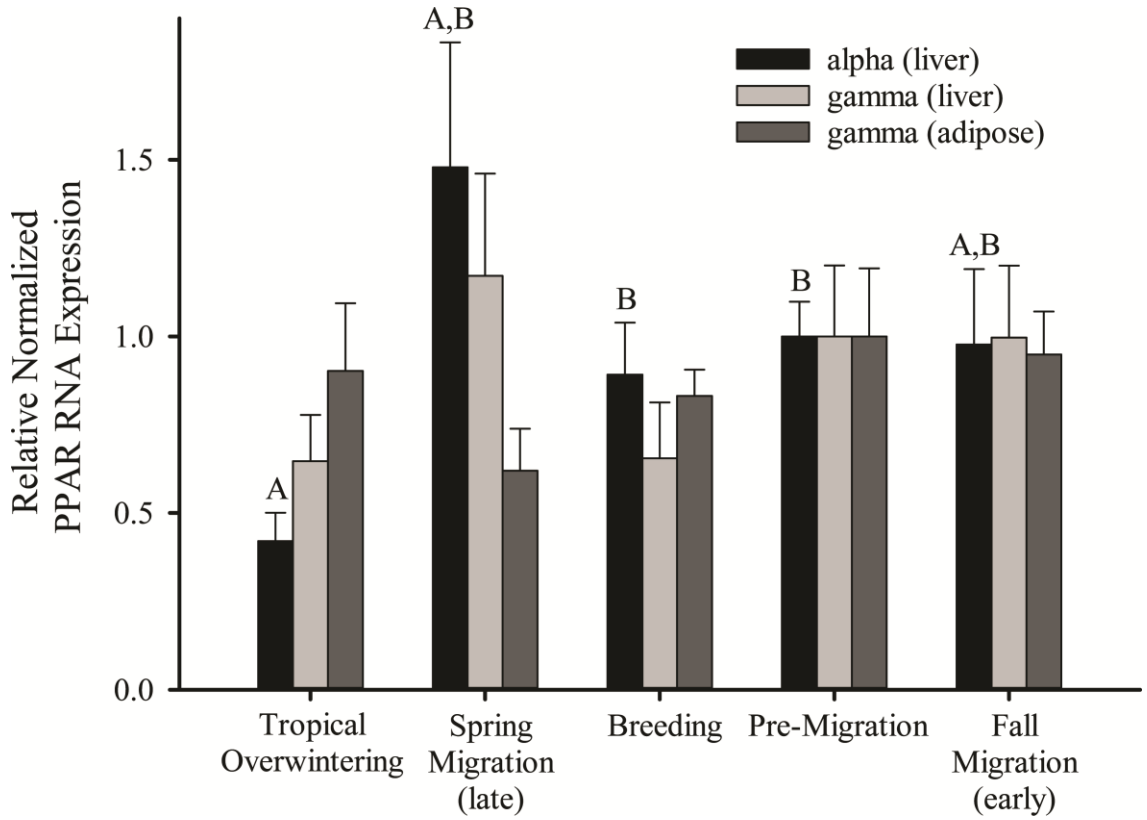

Figure 5.

Seasonal variation in PPAR expression in the liver and adipose tissues of gray catbirds. Expression of PPARα in the liver varied significantly across stages and was higher during spring migration compared to tropical overwintering. PPARγ expression showed no variation across stages in the liver or adipose tissue. Expression of each transcript was normalized with 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 mean ± SE.

Tables

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'

TABLE 2. *Seasonal variation in lipid content of catbird livers*

	Spring migration	Breeding	Pre-migration	Fall migration	*P*
	n = 5	*n = 2*	*n = 5*	*n = 4*	
% lipid	8.32 ± 1.67	7.11 ± 0.99	7.46 ± 0.90	6.67 ± 1.11	0.83

TABLE 3. *Seasonal variation in expression of liver tissue PPAR targets*

Gene	Tropics	Spring migration	Breeding	Pre-migration	Fall migration	*P*
	n = 10	*n = 14*	*n = 12*	*n = 18*	*n = 12*	
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

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

TABLE 4. *Seasonal variation in expression of adipose tissue PPAR targets*

Gene	Tropics	Spring migration	Breeding	Pre-migration	Fall migration	*P*
	n = 10	*n = 7*	*n = 5*	*n = 10*	*n = 12*	
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

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

TABLE 5. *Relationship of adipose tissue (a) lipase and perilipin expression to the rate of glycerol release or of target genes (tissue noted as 'a' for adipose or 'l' for liver) to PPAR gene expression or to one another (LPL only).*

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. PPARG (a)	44	0.111	0.027*
PLIN3 (a) vs. PPARG (a)	44	0.081	0.0620
LPL (a) vs. PPARG (a)	44	0.232	0.0009*
HSL (a) vs. PPARG (a)	44	0.070	0.083
LPL (l) vs. PPARA (l)	66	0.019	0.270
Lpl (l) vs. Lpl (a)	42	0.0002	0.936

Note: Asterisks indicate a significant linear relationship.