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RESEARCH ARTICLE

PPAR expression, muscle size and metabolic rates across the gray catbird's annual cycle are greatest in preparation for fall migration

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ABSTRACT

Phenotypic flexibility across the annual cycle allows birds to adjust to fluctuating ecological demands. Varying energetic demands associated with time of year have been demonstrated to drive metabolic and muscle plasticity in birds, but it remains unclear what molecular mechanisms control this flexibility. We sampled gray catbirds at five stages across their annual cycle: tropical overwintering (January), northward spring (late) migration (early May), breeding (mid June), the fall pre-migratory period (early August) and southward fall (early) migration (end September). Across the catbird's annual cycle, cold-induced metabolic rate ($\dot{V}_{O_2summit}$) was highest during migration and lowest during tropical wintering. Flight muscles exhibited significant hypertrophy and/or hyperplasia during fall migratory periods compared with breeding and the fall pre-migratory period. Changes in heart mass were driven by the tropical wintering stage, when heart mass was lowest. Mitochondrial content of the heart and pectoralis remained constant across the annual cycle as quantified by aerobic enzyme activities (CS, CCO), as did lipid catabolic capacity (HOAD). In the pectoralis, transcription factors PPARα, PPARδ and ERRβ, coactivators PGC-1α and β, and genes encoding proteins associated with fat uptake (FABPpm, Plin3) were unexpectedly upregulated in the tropical wintering stage, whereas those involved in fatty acid oxidation (ATGL, LPL, MCAD) were downregulated, suggesting a preference for fat storage over utilization. Transcription factors and coactivators were synchronously upregulated during pre-migration and fall migration periods in the pectoralis but not the heart, suggesting that these pathways are important in preparation for and during early migration to initiate changes to phenotypes that facilitate long-distance migration.

KEY WORDS: Avian migration, Metabolic flexibility, $\dot{V}_{O_2summit}$, Peroxisome proliferator-activated receptors, Pectoralis

INTRODUCTION

The annual cycle of a migratory bird is composed of distinct life history stages (i.e. breeding, pre-migration, migration, wintering) that are characterized by a specific set of environmental variables, energetic demands, behaviors and corresponding physiological traits (Ramenofsky et al., 2012; Wingfield, 2005). To fully characterize the challenges of life stage transitions, data across the full annual cycle are essential (Marra et al., 2015). Rapid and reversible changes in physiology are required to adjust to the variation in energy expenditure across the annual cycle to maintain maximal performance in each stage (Jacobs and Wingfield, 1999; Ramenofsky et al., 2012; Ricklefs and Wikelski, 2002; Wingfield, 2005). Such coordinated physiological flexibility requires precise regulation; however, the mechanisms mediating these changes remain poorly known.

The migratory stages appear to present the most significant energetic challenges of the avian annual cycle, as the demands of extended non-stop flights drive metabolic capacity to its highest levels (Corder and Schaeffer, 2015; Swanson, 1995, 2010; Swanson and Dean, 1999). Migration is an energetically demanding life history stage due to the high cost of the intense flights required to reach wintering or breeding grounds (Wikelski et al., 2003), and is associated with characteristic phenotypic remodeling (Bauchinger and Biebach, 2001; Dietz et al., 1999; Marsh, 1981). In contrast, it is proposed that overwintering in tropical locations relaxes metabolic demand as birds experience warm temperatures and abundant food supply without the energetic costs of defending territories (Ramenofsky et al., 2012). Indeed, energy expenditures in wintering migrants may approximate the levels found in tropical residents (Ricklefs and Wikelski, 2002).

Physiological alterations in structure and/or function that facilitate extended migratory flight include an elevated metabolic rate to increase energetic capacity during migration (Corder and Schaeffer, 2015; Swanson, 1995, 2010; Swanson and Dean, 1999), flight muscle hypertrophy to provide increased power (Bauchinger and Biebach, 2001; Dietz et al., 1999; Lindstrom et al., 2000; Marsh, 1984; Piersma, 1998; Vezina et al., 2006), and cardiac hypertrophy to supply oxygen to working muscles (Lindstrom et al., 2000; Piersma, 1998). Flight muscle remodeling includes an increased activity of the oxidative metabolic enzymes [citrate synthase (CS) and cytochrome *c* oxidase (CCO)] and the enzyme involved in fatty acid oxidation [3-hydroxyacyl-co A dehydrogenase (HOAD)] (Dick, 2017; Guglielmo et al., 2002; Lundgren and Kiessling, 1985, 1986; Marsh, 1981). Oxidative enzyme activities can increase during flightless periods, suggesting that endogenous regulation occurs. CS activity increases in preparation for migration in blue-winged teals (*Anas discors*) during the flightless molting period (Saunders and Klemm, 1994). However, it remains unclear whether flapping flight further increases oxidative enzyme activities. Flight did not influence the activities of CS, HOAD or carnitine palmitoyl transferase (CPT) in the pectoralis of migratory yellow-rumped warblers (*Setophaga coronata*) flown in a wind tunnel compared

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List of symbols and abbreviations

ACAD	fatty acyl-CoA dehydrogenase
ATGL	adipose triglyceride lipase
CCO	cytochrome *c* oxidase
CD36	fatty acid translocase
CPT	carnitine palmitoyl transferase
CS	citrate synthase
ERR	estrogen-related receptor
FABPpm	plasma membrane fatty acid binding protein
FATP1	fatty acid transport protein 1
HOAD	3-hydroxyacyl-coA dehydrogenase
HSL	hormone sensitive lipase
LPL	lipoprotein lipase
MCAD	medium-chain acyl-CoA dehydrogenase
NR	nuclear receptor
PGC-1	peroxisome proliferator-activated receptor gamma coactivator 1
PLIN1	perilipin 1
PLIN3	perilipin 3
PPAR	peroxisome proliferator-activated receptor
PPRE	PPAR response element
PUFA	polyunsaturated fatty acid
qRT-PCR	reverse transcription quantitative PCR
RER	respiratory exchange ratio
RPLP0	ribosomal protein lateral stalk subunit P0
RXR	9-cis-retinoic acid receptor
$\dot{V}_{O_2summit}$	sustained metabolic rate during a cold challenge

with unflown birds (Dick, 2017). However, life history stage did determine oxidative capacity of the pectoralis, as birds in a fall migratory condition had higher enzyme activities compared with birds maintained on a winter light cycle (Dick, 2017).

Peroxisome proliferator-activated receptors (PPARs) are potential mediators of seasonal changes in avian metabolism. PPARs are ligand activated nuclear receptor (NR) transcription factors that bind as heterodimers with the 9-cis-retinoic acid receptor (RXR) to PPAR response elements (PPREs) located on promoter regions of genes (Reddy and Hashimoto, 2001; Wang, 2010). The binding of fatty acid ligands recruits cofactors, including PPARγ coactivator-1 alpha and beta (PGC-1α and PGC-1β), to the PPAR complex, and activates the transcription of specific target genes (Reddy and Hashimoto, 2001; Wang, 2010). PPARs regulate many aspects of muscle metabolism in mammals, and the expression patterns of each isoform (PPARα, PPARδ and PPARγ) reflect their major regulatory roles. We recently demonstrated that these isoforms are all expressed in catbirds, respond to the same fatty acid ligands, and activate genes encoding fatty acid utilization proteins (LPL and CPT1b) (Hamilton et al., 2018). In mammals, PPARα is expressed mainly in oxidative tissues such as skeletal muscle, heart and liver, and it regulates target genes involved in lipid oxidation such as fatty acyl-CoA dehydrogenases (ACADs) and carnitine palmitoyltransferase 1B (CPT1) (Bensinger and Tontonoz, 2008). PPARδ (also referred to as PPARβ) is highly expressed in skeletal muscle and heart, regulates target genes involved in mitochondrial respiration, and overlaps with PPARα to target fatty acid uptake (e.g. CD36) and oxidation (e.g. CPT1) (Ehrenborg and Krook, 2009). PPARγ is highly expressed in adipose tissue and controls the expression of genes encoding proteins involved in lipid transport, such as fatty acid binding protein (FABPpm) and fatty acid translocase (CD36), and in lipolysis, adipose triglyceride lipase (ATGL) and lipoprotein lipase (LPL) (Bensinger and Tontonoz, 2008; Wang, 2010). Another group of NRs potentially involved in regulating avian muscle metabolism are the estrogen-related receptors (ERRα, ERRβ, ERRγ) that are also coactivated by PGC-1α and PGC-1β (Alaynick, 2008; Huss et al., 2004). In mammals, ERRα and ERRγ are ubiquitously expressed in oxidative tissues, such as heart and skeletal muscle, and are important regulators of fatty acid uptake and mitochondrial oxidation, mediating their effects directly via target gene activation and indirectly through regulation of PPARα expression (Alaynick, 2008; Huss et al., 2004). ERRβ has a more restricted expression pattern than the other isoforms, but it has been implicated in metabolic function (Huss et al., 2015). Data from RNA-sequencing revealed that all three PPARs as well as the ERRβ and ERRγ isoforms are expressed in birds (Hamilton et al., 2018). Based on the high degree of structural and functional conservation between mammalian and avian PPAR and ERR proteins (Dick, 2017; Hamilton et al., 2018), we predict that these NRs and their coactivators are involved in flight muscle and cardiac metabolic remodeling throughout the life cycle.

The present study characterized the phenotypic changes in an avian migrant across the annual cycle at the organismal, tissue and molecular levels to elucidate the regulatory mechanisms responsible for seasonal metabolic flexibility. The aim of this study was to determine the potential roles of the NRs PPARα, PPARδ, ERRβ and ERRγ, and their coactivators, PGC-1α and PGC-1β, in regulating seasonal phenotypic plasticity of the pectoralis muscle and heart in gray catbirds (*Dumetella carolinensis*). To identify the regulatory roles of PPARs and ERRs in free-living songbirds, we tested the hypothesis that PPARs, ERRs and PGC-1 coactivators are strongly associated with each other and with the selected metabolic genes. We predicted that metabolic genes directly transcribed by PPARs and ERRs would be strongly associated with the NRs. We also tested the hypothesis that expression of PPARs, ERRs and their target genes is upregulated during migratory periods compared with non-migratory periods, and that the activation of these molecular pathways increases the metabolic capacity of the flight muscle and heart. We predicted that gene expression of metabolic enzymes and their upstream regulators correlates with changes in tissue biochemical properties and whole-organismal metabolic rates across the annual cycle.

MATERIALS AND METHODS

Animals and experimental design

The gray catbird [*Dumetella carolinensis* (Linnaeus 1766); hereafter 'catbird'] is a migratory songbird that breeds in north, central and eastern North America. Catbirds winter in southern North America and in Central America. High catbird densities in Ohio and Belize facilitated capture during all sampling periods.

We characterized phenotypic traits at five stages across the catbird annual cycle: tropical wintering (5–20 January), northward spring (late) migration (30 April–11 May), breeding (11–27 June), the fall pre-migratory period (5–15 August) and southward fall (early) migration (17 September–3 October). During both migrations, birds were captured in Ohio; thus we considered that birds in the spring were in a late migratory state because they had completed the majority of their migration. Likewise, we considered birds to be in an early migratory state during the fall because birds had from 1000 to 2500 km of migratory flight remaining. The sampling period for each stage lasted approximately 2 weeks and was separated from adjacent sampling periods by at least 1 month to reduce any potential carryover effects. Catbirds were captured at each stage using mist-nets between sunrise and early afternoon in either Hueston Woods State Park (39°34′N, 84°44′W) or the Miami University Ecology Research Center (39°30′N, 84°45′W) near Oxford, Ohio, USA, and in Indian Church Village in Belize (17°45′N, 88°40′W). For cold-induced metabolic rate ($\dot{V}_{O_2summit}$)

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measurements, we used n=10–13 catbirds per stage, all of which were released after measurements were made. For subsequent measurements, we used a combination of catbirds caught specifically for this study and unpublished data collected as part of a previous study (Corder et al., 2016). For body mass and body composition we used n=20–56 or n=14–21 catbirds per stage. Of these, catbirds that were not used for tissue masses were released after measurements. For tissue masses, we used n=10–26 catbirds per stage. For functional assays, we used n=9–11 catbirds per stage. Note that this last category represents the animals that were killed for this study. In all cases, after capture, catbirds were weighed to the nearest 0.01 g using a microbalance (OHAUS, Scout Pro, Parsippany, NJ, USA). Body mass and body composition data (Table 1) for catbirds captured during the annual cycle were reported previously for the years 2012 and 2013 (Corder et al., 2016); however, Table 1 includes data from the years 2009 and 2012–2014. All birds in Ohio were analyzed for whole-body composition using an EchoMRI SuperFLEX™ analyzer (EchoMRI, Houston, TX, USA). This nonlethal quantitative magnetic resonance technique to measure total lean and fat masses was previously validated in songbirds (Guglielmo et al., 2011).

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, the US Fish and Wildlife Service 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.

Organismal metabolism

$\dot{V}_{O_2summit}$ (maximal shivering oxygen consumption during an acute cold stress) was measured at each of the five life cycle stages following Rosenmann and Morrison (1974). Individual catbirds were placed in a sealed chamber (volume ∼1 liter) and the temperature of the chamber was decreased to 4°C using a refrigerator. Flow through the chamber (in push mode) was maintained at a constant flow rate of 1.2 l min^{-1} of a 21% oxygen/89% helium (HeO_2) gas mixture using a mass flow meter (0–5 l min^{-1}, Sierra Instruments, Monterey, CA, USA) controlled by a flow controller (model MFC-2, Sable Systems, Las Vegas, NV, USA). After the chamber, gases passed through the CO_2 analyzer (FoxBox Field Gas Analysis System, Sable Systems), a Drierite column and the O_2 analyzer (FoxBox). Outputs from the flow controller and gas analyzers were collected at a rate of one sample per second using the Expedata program (Sable Systems). Trials were terminated after 20 to 30 min once O_2 consumption had reached a steady-state plateau. Cloacal temperature was obtained immediately after each trial to verify that birds were hypothermic (body temperature ≤37°C), which indicated a maximal effort. $\dot{V}_{O_2summit}$ was calculated using the highest 1-min average gas concentration values and the equation from the Expedata manual (Sable Systems):

$$\dot{V}_{O_2summit} = f \cdot ((F_{I_{O_2}} - F_{E_{O_2}}) - F_{E_{O_2}} \cdot (F_{I_{CO_2}} - F_{E_{CO_2}}))/(1 - F_{E_{O_2}}), \quad (1)$$

where f is the flow rate (corrected to standard temperature, pressure and dry gas), $F_{I_{O_2}}$ and $F_{E_{O_2}}$ are the fractional content of oxygen in incurrent and excurrent air, respectively, and $F_{I_{CO_2}}$ and $F_{E_{CO_2}}$ are the fractional content of carbon dioxide in incurrent and excurrent air, respectively. We normalized ml of O_2 consumed per minute by dividing by individual body mass (g). The respiratory exchange ratio (RER) was calculated as the CO_2 produced/O_2 consumed. After each trial, birds were released after tail feathers were clipped to prevent measuring the same individuals twice per season.

Tissue and organ masses

Upon capture of a second set of catbirds in each life stage (except tropical overwintering), we determined fat and lean masses using an EchoMRI SuperFLEX™ body composition analyzer. For all stages, these birds were then euthanized with an overdose of inhaled isoflurane and tissue samples collected. The pectoralis (flight muscle), heart and gastrocnemius (leg muscle) were removed and immediately weighed. A subset of the tissues was preserved in Qiagen Allprotect Tissue Reagent and stored at 4°C for 1–2 days in Ohio and 4–10 days in Belize, and these samples were subsequently stored at −80°C to quantitate relative gene expression using real-time quantitative PCR (qRT-PCR). An additional subset of the tissues from all of the stages except tropical overwintering were flash-frozen in liquid nitrogen and stored at −80°C for biochemical and lipid composition analyses.

Mitochondrial and lipid oxidation assays

Enzyme activities of CS, CCO and HOAD were quantified in the pectoralis and heart. Samples were homogenized in nine volumes of homogenization buffer (200 mmol l^{-1} K_2HPO_4, 200 mmol l^{-1} K_2HPO_4, 10 mmol l^{-1} EDTA, 1 mol l^{-1} dithiothreitol, pH 7.5) on ice using a Power Gen 125 homogenizer (Fisher Scientific, Waltham, MA, USA) for 3×10 s bouts. Samples were centrifuged at 300 ***g*** for 10 min at 4°C. The supernatant was removed and samples were kept on ice and used immediately for CS and CCO, and frozen at −80°C for HOAD assays. CS and CCO protocols were modified from Houle-Leroy et al. (2000) and the HOAD protocol was modified from Price et al. (2010) as described below.

To determine CS activity, muscle homogenates were added to assay reagent (100 mmol l^{-1} Tris-HCl, pH 8.0) with an excess of 5,5′ dithiobis-2-nitrobenzoic acid (DTNB; 0.10 mmol l^{-1}), acetyl-CoA (0.15 mmol l^{-1}) and oxaloacetate (0.15 mmol l^{-1}). The reaction was monitored at 412 nm. To determine CCO activity,

Table 1. Gray catbird body mass, wet fat and lean masses (g) from individuals collected in Oxford, Ohio, USA, during fall pre-migration, fall migration, spring migration and breeding (2012–2014), and in Indian Church Village, Belize, during tropical overwintering (2014)

Body condition	Pre-migration	Fall migration	Tropical overwintering	Spring migration	Breeding	*P*
Sample size	30, 14	56, 50	20, 0	27, 21	23, 14	
Body mass	34.7±0.4^{a}	37.1±0.3^{b}	35.8±0.5^{a,b}	34.2±0.5^{a}	34.1±0.5^{a}	<0.001
Fat mass	0.93±0.1^{a}	2.7±0.7^{b}	–	2.4±0.4^{b}	0.77±0.2^{a}	<0.001
Lean mass	29.4±0.4^{a}	28.2±0.5^{a}	–	26.0±0.3^{b}	27.6±0.4^{a,b}	<0.001

Data are presented as means±s.e.m. Sample sizes correspond to body mass, fat/lean masses. Different letters indicate statistical differences in body condition among stages determined by one-way ANOVA and *post hoc* Tukey's test for body mass and Wilcoxon and Kruskal–Wallis rank sums test followed by Steel–Dwass pairwise comparisons for fat and lean masses.

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homogenates were added to assay reagent (100 mmol l^{-1} potassium phosphate, pH 7.5) with an excess of reduced cytochrome *c* (0.075 mmol l^{-1}). Cytochrome *c* was reduced by adding sodium hydrosulfite, and excess sodium hydrosulfite was removed by bubbling with compressed air for 30 min on ice. The reaction was monitored at 550 nm against a reference of 0.075 mmol l^{-1} cytochrome *c* oxidized with 0.33% potassium ferricyanide. To determine HOAD activity, homogenates were added to assay reagent (50 mmol l^{-1} imidozole, pH 7.4 at 39°C) with an excess of EDTA (1 mmol l^{-1}), NADH (0.225 mmol l^{-1}) and acetoacetyl CoA (0.1 mmol l^{-1}) and the reaction was monitored at 340 nmol l^{-1}.

Enzyme activity was calculated by obtaining the slope of the reaction (change in absorbance) in a temperature-controlled spectrophotometer (DXT880 Multimode Detector, Beckman Coulter, Fullerton, CA, USA) at 39°C over the course of 5 min. Extinction coefficients for each enzyme substrate are: 13.6×10^3 for DTNB, 19.1×10^3 for ferricytochrome *c* and 6.22×10^3 for NADH. All muscle homogenates were diluted to 1:100 and were run in duplicate in a final volume of 1 ml. Enzyme activity was calculated by subtracting a blank (control) run from the reactions (lacking substrates acetyl-CoA for CS, sample for CCO and acetoacetyl CoA for HOAD). Enzyme activity is expressed as units per gram of tissue, where one unit is equal to 1 µmol product min^{-1}.

Determination of tissue lipid content

Pectoralis, heart, gastrocnemius and liver samples from a subset of animals collected in Ohio were used to determine tissue lipid content. Lipids were extracted from 20–91 mg of tissue using a modified Folch method (Folch et al., 1957). After weighing, the frozen tissue piece was 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 a nitrogen stream using a NEVAP 111 (Organomation, Berlin, MA, USA). The remaining extract was weighed and the lipid content (as a percentage of original tissue wet mass) was obtained.

Gene expression analysis

Quantitative reverse transcription PCR (qRT-PCR) was performed to quantitate relative expression of PPARs, ERRs, PGCs and selected target genes. Total RNA was extracted from pectoralis muscle and heart (50 mg) using TRIzol® Reagent (Ambion, Life Technologies, Carlsbad, CA, USA). RNA concentrations and quality were verified using a NanoDrop (Nanodrop Technologies, Wilmington, DE, USA). RNA (1 µg) was reverse transcribed using the iScript cDNA synthesis kit (Bio-Rad, Hercules, CA, USA), and cDNA was used as template for qPCR. Each 15 µl PCR reaction mixture comprised cDNA template, 0.17 µmol l^{-1} gene-specific primers (primer-specific optimal concentration) and 2X iQ SYBR® Green Supermix (Bio-Rad). The temperature cycles for each 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 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. The real-time values were derived from a standard curve generated for each primer set. Primer sequences were derived directly from gray catbird RNA-seq data. (Hamilton et al., 2018). Primers in our study met the following criteria: (1) amplification of a single product indicated by a single peak in the melting curve analysis; (2) sequence of the PCR product confirming amplification from the proper gene; and (3) efficiency of amplification between 90 and 110%. In all cases, cycle threshold (C_t) values ranged from 18 to 25, except for Lipe and Fatp1, which were detected between 30 and 31, and PGC-1β, which was detected in the 32–33 range. Primer sequences are shown in Table S1. Transcript expression levels were normalized to the reference gene *RPLP0*, which codes for the ribosomal protein subunit P0 and is highly conserved across tissues and species, and did not vary across the catbird's annual cycle ($F_{3,34}$=0.64, P=0.64). Ribosomal genes are commonly used as a reference gene in birds. For example, *RPL13* was among the most stable normalization genes in chicken kidney cells (Batra et al. 2016). Similarly, *RPL13* and *RPL19* were validated in zebra finch, chicken and ostrich brain tissue (Olias et al. 2014). Experimental and control reactions were run independently (*RPLP0* was not multiplexed with each experimental run). The averages from triplicate PCR wells were used for correction. Transcript expression is reported relative to the pre-migratory stage.

Statistical analyses

Statistical analyses were conducted using JMP software (SAS Institute, version 10.0). The effect of annual stage on each measure was compared using a one-way ANOVA with a *post hoc* Tukey's HSD comparison. Data were checked for normality using Shapiro–Wilk's test and constant variance using a Levene's test. Those data that did not meet these assumptions were log transformed, although all figures present non-transformed data. Log transformation did not correct for normality and/or equal variance for all data sets as indicated in figure and table legends. In these cases, non-parametric statistics, Wilcoxon and Kruskal–Wallis tests, were conducted. Each pair of Student's *t*-tests were conducted to determine differences in oxidative and catabolic enzyme activities between the heart and pectoralis. The gene expression data normalized to the reference gene *RPLP0* (but not normalized to season) were used to perform a correlation analysis to measure the strength of association between gene pairs for the entire annual cycle. We used R 3.4.3 (https://www.r-project.org/) to conduct a Spearman's rank-order correlation analysis, the nonparametric equivalent of the Pearson's correlation, because the data violated the assumption of a normal distribution. We computed the correlation matrix (Fig. S1) for gene pairs using the Corrplot package and extracted Spearman's correlation coefficients and *P*-values using the Hmisc package. We used Cystoscape 3.7.1 (Shannon et al., 2003) to construct the gene network (see Fig. 4) using Spearman's correlation coefficients and *P*-values extracted from R (Tables S3 and S4).

RESULTS

Body mass and body composition

A subset of the data for body mass and composition across the annual cycle (Table 1) was reported previously (Corder et al., 2016). Based on the larger sample size studied here, we confirmed that catbirds maintained a constant body mass across most of the annual cycle with the exception of fall (early) migration, during which mass was significantly increased ($F_{4,152}$=11.41, P<0.001; Table 1). Body composition analysis revealed that fat mass was also significantly higher during migration compared with non-migratory stages (χ^2_4=26.25, P<0.001; Table 1). Lean mass was highest during pre-migration and fall, and lowest during spring (late) migration (χ^2_4=25.52, P<0.001; Table 1).

Organismal summit metabolic rate

There was a significant effect of annual stage on whole-body maximum metabolic rate ($\dot{V}_{O_2summit}$) and RER, an indirect measure of the relative contribution of fuels to oxidative energy metabolism.

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The $\dot{V}_{O_2summit}$ was lowest in the tropics (19–29% lower) and highest during fall (early) migration (χ^2_4=25.49, P<0.001; Fig. 1) compared with all other stages. The differences observed in these stages remained when $\dot{V}_{O_2summit}$ was normalized to body mass by dividing by individual body mass ($F_{4,49}$=6.04, P<0.0005). The RER displayed an opposite pattern, and was greatest in the tropical wintering stage (0.81) and lowest during fall migration (0.55) (χ^2_4=34.49, P<0.0001; Fig. 2).

Heart and skeletal muscle structure

The pectoralis muscle, predominantly relied on for flight, was significantly larger during fall (early) migration compared with pre-migration, but did not significantly differ in the other stages ($F_{4,73}$=3.04, P=0.02; Table 2). In contrast, pectoralis mass normalized to total lean mass was highest during spring migration (χ^2_3=30.24, P<0.001), and pectoralis mass normalized to body mass was highest during spring migration and breeding (χ^2_4=18.72, P<0.001; Table S2). Absolute heart mass was non-significantly highest during both migrations, but unlike the pectoralis, was significantly lowest during tropical overwintering ($F_{4,75}$=16.91, P<0.001; Table 2). Heart size scaled to body mass remained lowest in the tropics (χ^2_4=21.58, P<0.001; Table S2). As observed for the pectoralis, heart mass normalized to lean mass was highest during both migrations ($F_{4,75}$=22.32, P<0.001) and when normalized to whole body mass was highest during spring migration (Table S2). Although not significant, gastrocnemius (leg) mass tended to be lowest during spring migration ($F_{4,64}$=2.42, P=0.06; Table 2).

Skeletal muscle metabolism

There were no differences in metabolic enzyme activities among life history stages per gram of tissue in the pectoralis (CS, χ^2_3=1.38, P=0.71; CCO, $F_{3,39}$=0.04, P=0.99; HOAD, χ^2_3=4.90, P=0.18; Table 3) or for the total activity of the whole muscle mass (wet mass) (CS, χ^2_3=4.35, P=0.23; CCO, χ^2_3=1.95, P=0.58; HOAD, χ^2_3=6.68, P=0.08) or in the heart per gram of tissue (CS, $F_{3,32}$=1.89, P=0.15; CCO, χ^2_3=1.381, P=0.73; HOAD, χ^2_3=5.35, P=0.15; Table 3) or for the total activity of the whole organ mass (CS, χ^2_3=2.64, P=0.45; CCO, $F_{3,34}$=0.31, P=0.82; HOAD, $F_{3,34}$=2.07, P=0.125). Analysis of mitochondrial enzyme activity revealed differences between cardiac and flight muscle oxidative capacity. CS and CCO activities, measures of mitochondrial abundance, were 52% and 53% greater, respectively, in the heart compared with in the pectoralis (CS, $F_{1,77}$=92.51, P<0.0001; CCO, $F_{1,78}$=45.54, P<0.0001; Table 3). HOAD activity was 30% greater in the pectoralis compared with in the heart ($F_{1,70}$=14.47, P=0.0003; Table 3), indicating a greater ability to oxidize fatty acids in the flight muscle.

The lipid content of pectoralis, heart, gastrocnemius and liver did not differ across migration status. Between tissues, the heart had a higher percent lipid content compared with the pectoralis or liver at both non-migratory and migratory stages ($F_{3,57}$=4.14, P=0.01; Fig. 3).

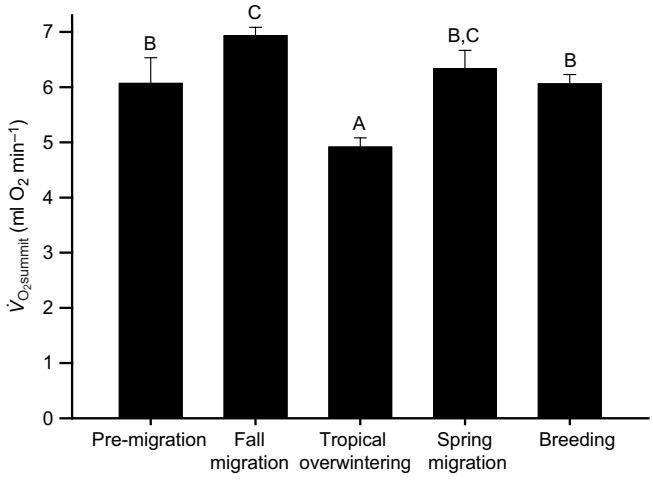

Fig. 1. Seasonal variation in cold-induced metabolic rate ($\dot{V}_{O_2summit}$). Mean (±s.e.m.) summit respiration measured at each of the defined life stages (n=13 for fall migration, n=10 for all other stages). Different letters indicate significance differences among stages determined by Wilcoxon and Kruskal–Wallis rank sums test and Steel–Dwass pair-wise comparisons.

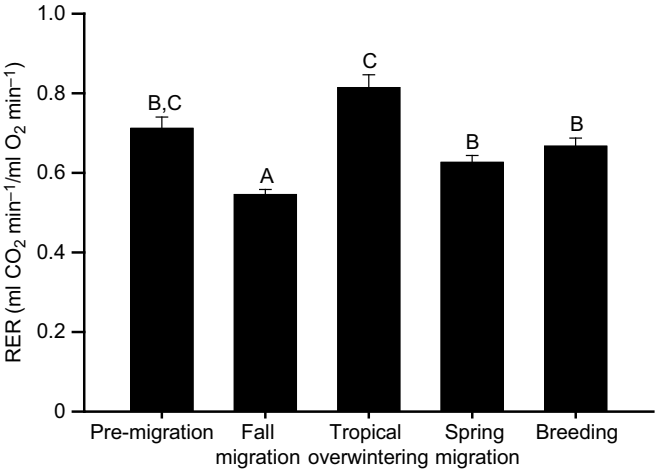

Fig. 2. Seasonal variation in the respiratory exchange ratio (RER). RER (mean±s.e.m., calculated as rate of CO_2 released/O_2 consumed) during $\dot{V}_{O_2summit}$. Sample sizes as in Fig. 1. Different letters indicate significance between RER among stages determined by Wilcoxon and Kruskal–Wallis rank sums test and Steel–Dwass pair-wise comparisons.

PPARs as metabolic regulators in the pectoralis, but not the heart

The NRs PPARα, PPARδ and ERRβ were highly correlated with each other and their coactivators PGC-1α and PGC-1β in catbird pectoralis (r_S=0.34–0.56, P<0.05; Fig. 4, Table S3, Fig. S1). ERRγ was only significantly correlated with PPARα (r_S=0.3, P=0.04). PPARα, PPARδ, ERRβ, PGC-1α and PGC-1β were directly associated with the genes that transcribe proteins involved in mitochondrial metabolism: *CS* (r_S=0.27–0.33, P<0.07) and *MCAD* (r_S=−0.26 to −0.37, P<0.1). We expected metabolic transcription factors to be positively correlated with MCAD, the enzyme that catalyzes the initial step of the mitochondrial fatty acid beta-oxidation pathway; however, this association was consistently negative. PPARα, ERRβ, PGC-1α and PGC-1β were directly associated with the lipid droplet Plin3 (r_S=0.35 to 0.57, P<0.05). NRs were inconsistently associated with genes involved with fat transport and uptake, i.e. *CD36* (PPARα, r_S=0.26, P=0.09; ERRγ, r_S=−0.26, P=0.09), *FATP1* (ERRβ, r_S=0.29, P=0.06) and *FABPpm* (ERRγ, r_S=0.25, P=0.1). However, none of the regulators were directly associated with genes involved in fat catabolism (*LPL*, *HSL*, *ATGL*).

In the catbird pectoralis muscle, the relative gene expression of PPARα, PPARδ, ERRβ and PGC-1α, PGC-1β, and several selected target genes involved in muscle oxidative pathways and fatty acid transport and oxidation were significantly different among stages of

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Table 2. Gray catbird absolute tissue mass (g) from individuals collected in Oxford, Ohio, USA, during fall pre-migration, fall migration, spring migration and breeding (2012–2014), and in Indian Church Village, Belize, during tropical overwintering (2014)

Tissue	Pre-migration	Fall migration	Tropical overwintering	Spring migration	Breeding	*P*
Sample size	17	14	10	26	10	
Pectoralis mass (g)	4.53±0.10[a]	4.87±0.06[b]	4.51±0.13[a,b]	4.66±0.06[a,b]	4.48±0.08[a]	0.02
Sample size	19	14	10	27	9	
Heart mass (g)	0.31±0.01[a]	0.34±0.01[a]	0.25±0.01[b]	0.33±0.01[a]	0.31±0.01[a]	<0.001
Sample size	17	13	10	18	10	
Gastrocnemius mass (g)	0.26±0.01	0.27±0.01	0.26±0.01	0.23±0.01	0.25±0.01	0.06

Data are presented as means±s.e.m. Different letters indicate statistical differences of tissue mass among stages determined by one-way ANOVA and *post hoc* Tukey's test for heart and gastrocnemius and Wilcoxon and Kruskal–Wallis rank sums test followed by Steel–Dwass pairwise comparisons for pectoralis mass.

the annual cycle (Fig. 5, Table 4). Expression patterns in the tropical wintering stage were frequently the most divergent from the other stages, and expression in this stage was either significantly upregulated (PPARα, $F_{4,44}$=5.17, P=0.002; PGC-1α, $F_{4,44}$=5.82, P<0.001; PGC-1β, χ^2_4=6.50, P<0.001; ERRβ, $F_{4,44}$=5.00, P=0.002; FABPpm, $F_{4,44}$=3.71, P=0.01; Plin3, $F_{4,43}$=7.42, P=0.0002) or downregulated (ATGL, $F_{4,40}$=2.67, P=0.05; LPL, $F_{4,43}$=3.30, P=0.02; MCAD, $F_{4,44}$=3.28, P=0.02). When excluding the tropical wintering stage, the expression changes in PPAR and ERR transcription factors and targets were largely observed in pre-migration, fall (early) migration and spring (late) migration stages. The expression of PPARδ and fatty acid transporters LPL and CD36 was highest during pre-migration (PPARδ, $F_{4,44}$=3.88, P=0.01; CD36, $F_{4,44}$=2.87, P=0.04), the PGC-1 cofactors were upregulated during pre-migration and fall migration, and the expression of PPARα was highest during fall migration. The perilipins were upregulated during both fall and spring migration (Plin2, $F_{4,42}$=3.19, P=0.02), and MCAD and HSL were upregulated during spring migration (HSL, $F_{4,43}$=3.03, P=0.03). Finally, no expression changes were observed among the stages for the transcription factor ERRγ or the metabolic target genes *FATP1* and *CS* (ERRγ, $F_{4,44}$=1.05, P=0.40; *CS*, $F_{4,44}$=2.46, P=0.06; *FATP1*, χ^2_4=1.89, P=0.13). In the heart, there were no differences in gene expression levels in any of the target genes measured among the stages (*CD36*, $F_{4,40}$=1.78, P=0.15; *FATP1*, $F_{4,39}$=0.71, P=0.59; *FABPpm*, $F_{4,42}$=1.28, P=0.29; *MCAD*, χ^2_4=3.57, P=0.47; *CS*, $F_{4,41}$=0.79, P=0.54; Table 5); therefore, we did not measure transcription factor gene expression or perform correlation analyses.

DISCUSSION

Migratory birds must enhance metabolic output and increase muscle size to accomplish the energetically demanding long-distance migratory flight, a process that is then reversed during non-migratory stages (Corder and Schaeffer, 2015; Marsh, 1984; Swanson, 1995). As expected, we found that catbirds demonstrated phenotypic flexibility at all levels of organization examined that matched the demands of migration. Although PPAR and ERR expression tracked metabolic and fatty acid metabolic enhancements to some degree, their contribution appears to be one among many factors driving the migratory phenotype.

Like other neotropical migrant species, catbird metabolic capacity was highest during migration (Corder and Schaeffer, 2015; Swanson, 1995; Swanson and Dean, 1999), which likely facilitates endurance flight (Swanson, 2010). In some species, metabolic capacity was highest in spring migrants, and it was suggested that colder temperatures (Swanson and Dean, 1999) and faster rates of migration (Swanson, 1995) in the spring may drive elevated metabolic capacity compared with fall migration. However, the catbirds in our study exhibited similar $\dot{V}_{O_2summit}$ during spring and fall migrations. Previous studies have demonstrated higher metabolic rates in resident bird species from high latitudes compared with related tropical species (Wagner et al., 2013; Wiersma et al., 2007a, b), thought to reflect the 'pace of life' in each environment (Ricklefs and Wikelski, 2002). The first study to measure metabolic capacity of songbird migrants (northern waterthrush) at both tropical and temperate locales (Corder and Schaeffer, 2015) found that although $\dot{V}_{O_2summit}$ was highest during migration, rates during tropical wintering and temperate breeding were similar. We also found that catbird metabolic rates were elevated during migration. However, we found that it was lowest in the tropics, suggesting that catbirds adjust metabolic rates to match the reduced energetic demands of tropical residents. It is unlikely that lower $\dot{V}_{O_2summit}$ in the tropics is a strategy to avoid heat stress because average temperatures experienced by catbirds in Belize (15–30°C) were similar to temperatures in their northern breeding range in Ohio (16–28°C). Thus, while metabolic flexibility in migratory birds appears to be universal, the pattern varies by species. More detailed studies across the entire annual cycle and studies examining the tropical environment or specific life history variables, such as rate of migration, are needed to define the mechanisms underlying the differences among species.

The increase in systemic fat metabolism, indicated by lower RER, in parallel with increased metabolic capacity may reflect regulation of internal metabolic pathways, a shift to diets rich in fat during migration, or a combination of both. Given that birds have been found to show unusually low RER values (Walsberg and Wolf, 1995), the percent reliance of lipid substrates may be

Table 3. Gray catbird oxidative enzyme activities (μmol min^{-1} g tissue^{-1}) in the heart and pectoralis from individuals collected in Oxford, Ohio, USA, during fall pre-migration, fall migration, spring migration and breeding (2012–2014)

Tissue	Enzyme	Pre-migration	Fall migration	Spring migration	Breeding	*P*
Pectoralis	Citrate synthase	253.4±24.1 (11)	269.7±24.7 (11)	293.3±18.2 (10)	277.0±11.8 (10)	0.71
	Cytochrome *c* oxidase	753.8±34.8 (10)	653.9±28.0 (11)	716.5±55.9 (9)	667.3±24.8 (10)	0.98
	HOAD*	182.3±20.1 (9)	197.5±19.4 (9)	224.1±18.7 (9)	164.3±15.9 (8)	0.18
Heart	Citrate synthase*	412.8±23.5 (8)	371.1±19.7 (9)	415.8±19.2 (9)	436.1±18.4 (9)	0.15
	Cytochrome *c* oxidase*	1094.5±103.7 (8)	1095.3±93.5 (8)	1104.7±137.5 (9)	1037.6±67.3 (10)	0.73
	HOAD	131.4±15.6 (8)	134.4±8.9 (9)	173.8±16.8 (8)	154.4±13.0 (10)	0.15

Data are presented as means±s.e.m. (sample sizes, n=8–11). Asterisks indicate statistical differences in enzyme activity between pectoralis and heart muscles as determined by each pair, Student's *t*-test comparisons.

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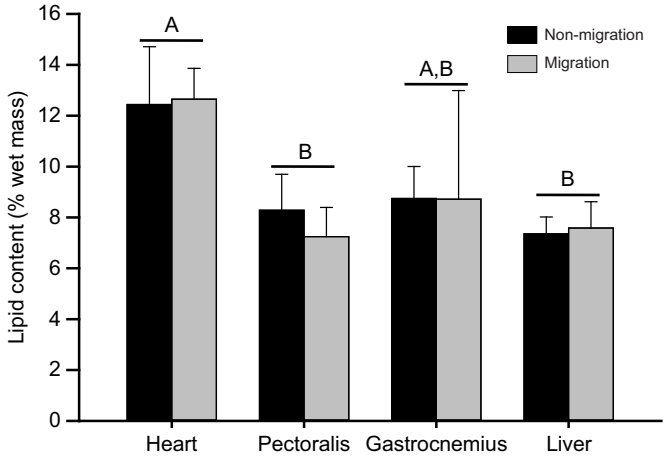

Fig. 3. Seasonal variation in percent lipid content. Mean (±s.e.m.) total lipid content in each tissue was measured in the heart, pectoralis, gastrocnemius and liver. Non-migration include birds during breeding (n=2) and pre-migration (n=5), while migration includes birds during spring (n=5) and fall migration (n=4). Different letters indicate significant differences in lipid content between the heart, pectoralis, gastrocnemius and liver determined by one-way ANOVA and pair-wise comparisons.

overestimated from this measure. We discuss evidence for internal modulation of metabolic pathways in response to dietary lipids and metabolic demand below. Regarding a shift in dietary substrate supply, several studies found that many migrating songbirds select diets rich in berries containing high amounts of lipids and antioxidants during migration, while in non-migratory periods they prefer protein-based insect diets (Bairlein and Gwinner, 1994; McWilliams et al., 2004; Parrish, 1997). Catbirds preferentially use habitats dominated by native shrubs during migration (Oguchi et al., 2018) and have better body condition in those habitats (Oguchi et al., 2017). We found that berries from these shrubs contain higher fat content than exotics (P.J.S., unpublished data). Furthermore, these diet preferences may enhance metabolic performance by reducing oxidative damage via antioxidants (Alan and McWilliams, 2013; Bolser et al., 2013; Cooper-Mullin and McWilliams, 2016) or by maximizing fat stores for use during endurance flight (Dick, 2017; Guglielmo et al., 2002; Pierce et al., 2005; Pierce and McWilliams, 2005). The effects of dietary variation on metabolic performance, muscle oxidative capacity and fatty acid use are not well understood.

As expected, to support migratory flight, the pectoralis muscles and heart were largest in absolute mass during the migratory period, similar to data from other migratory songbirds (King et al., 2015; Marsh, 1984) and shorebirds (Petit and Vezina, 2014; Vezina et al., 2006), as well as in winter cold-acclimated birds (Sgueo et al., 2012; Swanson et al., 2013). Although not measured in the same individuals, the average pectoralis mass was greatest during fall migration, when $\dot{V}_{O_2summit}$ was highest, in agreement with previous studies (Liknes and Swanson, 2011b; Sgueo et al., 2012; Swanson et al., 2013; Vezina et al., 2006). Similarly, cardiac mass was largest

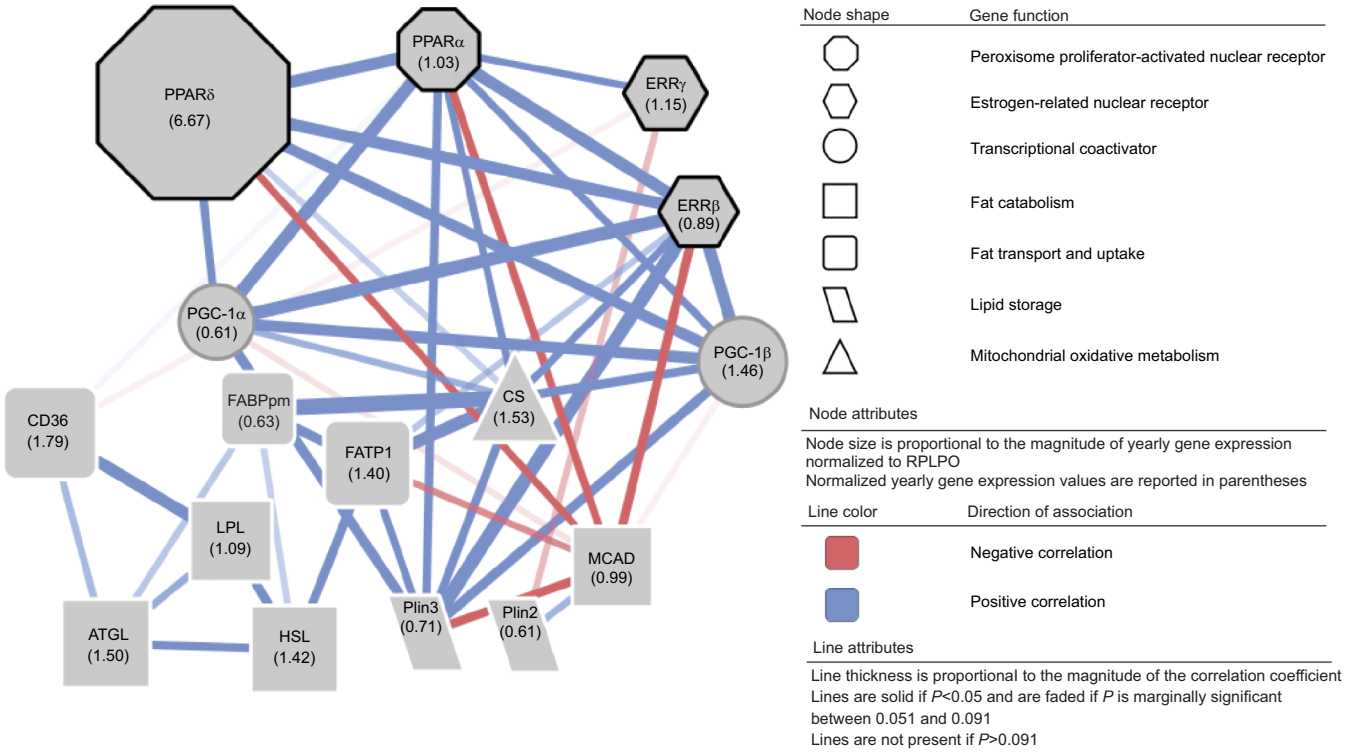

Fig. 4. Metabolic gene network of molecular regulation factors (PPARs, ERRs and PGCs outlined in black and gray) and their metabolic target genes (outlined in white) in gray catbird pectoralis. Each gene is represented by a node, and the size of the node is proportional to yearly gene expression levels normalized to *RPLP0*. Expression values are reported in parentheses within the nodes. N=45 for each gene. The shape of the nodes indicate a gene's main function. The spatial orientation of the network corresponds to similar functional roles and their degree of connectedness. The genes are connected by a solid line if the Spearman's correlation coefficient was statistically significant at P<0.05, and are connected by a faded line if P was marginally significant between 0.05 and 0.09. The thickness of the line is proportional to the magnitude of the correlation coefficient, so that coefficients closest to −1 and 1 have the thickest lines and coefficients close to 0 have the thinnest lines. Positive correlations are displayed in blue and negative correlations in red. Spearman's correlation coefficients were extracted in R 3.4.3 using the Hmisc package and the network was constructed using Cytoscape 3.7.1. See Tables S3 and S4 for a full list of Spearman's correlation coefficients and corresponding P-values.

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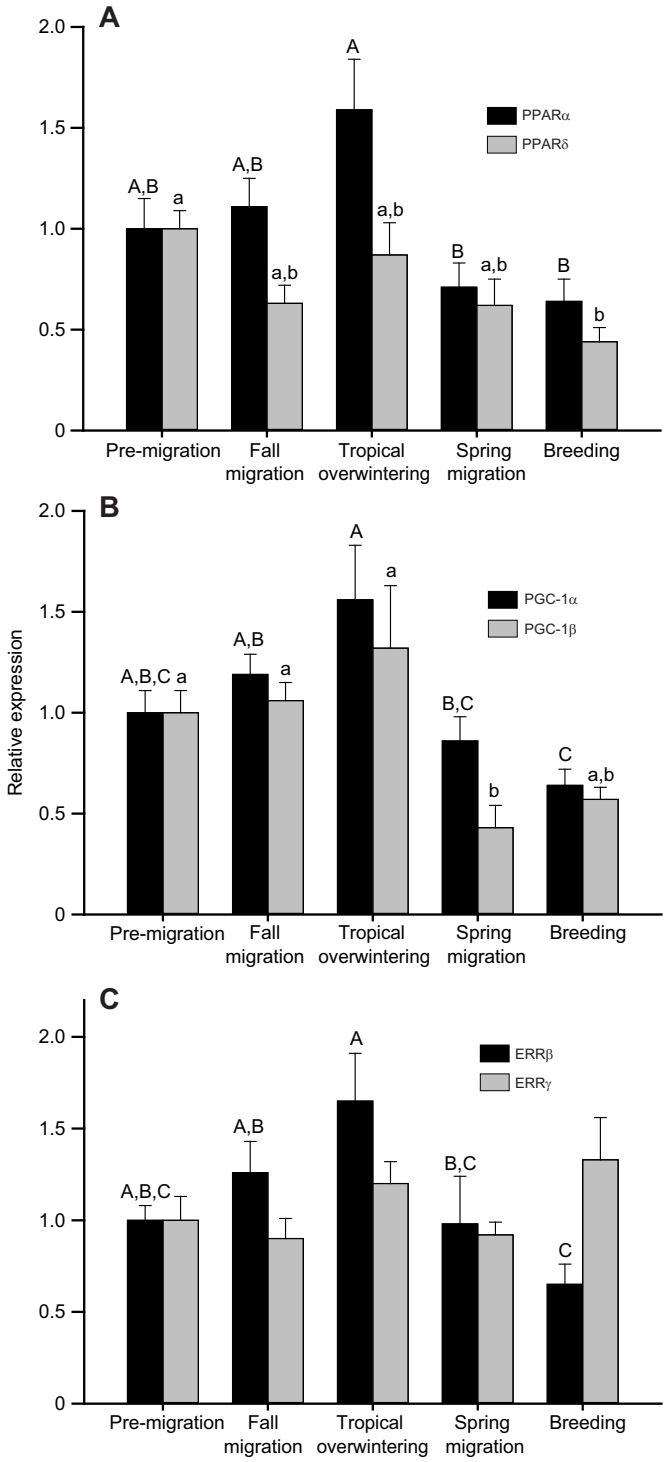

Fig. 5. Seasonal variation in gene expression. Quantitative real-time PCR analysis of transcripts encoding (A) nuclear receptors PPARα and PPARδ, (B) transcriptional coactivators PGC-1α and PGC-1β and (C) nuclear receptors ERRβ and ERRγ, all normalized to the housekeeping gene *RPLP0*. The mean (±s.e.m.) log-transformed values are reported. Different letters indicate significant differences between stages following a one-way ANOVA and pair-wise comparisons (n=10 during pre-migration, n=9 fall migration, n=7 tropics, n=10 spring migration, n=8 breeding for each gene).

during migratory phases, in agreement with previous work (e.g. King et al., 2015), presumably to support enhanced metabolic demand during endurance flight. For 457 species compared across a tropical-to-temperate life history divide, it was found that tropical birds have smaller pectoralis and heart sizes (Wiersma et al., 2012). In our study, heart mass was significantly lower during tropical wintering, although pectoralis size did not change in the transition from fall migration to the tropics, suggesting a disconnect between the timing of phenotypic change for these tissues. In contrast to the pectoralis, the gastrocnemius mass remained constant across the annual cycle, but was at its lowest during spring (late) migration (nearly significant at P=0.06). Total lean mass was significantly lower in the spring (with a nearly identical reduction as the gastrocnemius alone), supporting the observation that migrating birds catabolize proteins in non-critical tissues during flight. Similarly, other long-distance migrants (e.g. garden warblers, *Sylvia borin*) reduce their leg muscle mass by 19% during migratory flight (Bauchinger and Biebach, 2001; Biebach, 1998), and thrush nightingale (*Luscinia luscinia*) used protein as 10% of their energy source during a wind tunnel simulated migration (Klassen et al., 2000).

Enhanced cellular metabolic and lipid oxidation capacities may serve to improve metabolic performance during seasonal climatic variation and migration, as proposed by Swanson (2010) and based on data from long- and short-distance migrant songbird species (Lundgren and Kiessling, 1985) and winter-acclimatized birds (Liknes and Swanson, 2011a). However, CS and CCO activities remained unchanged in catbird pectoralis and heart across all stages (Table 3). Similarly, Marsh (1981) found activities of CS and CCO to be similar in catbird pectoralis, supracoracoideus and heart in Florida fall migrants and Michigan summer residents. However, fatty acid oxidation in those migrants, as measured by HOAD activity, was double that of the summer residents. In our study, although not statistically significant, HOAD activities in both pectoralis and heart tended to be higher during migratory stages (Table 3). Thus, while metabolic challenge leads to modification of muscle metabolic enzyme activities in some bird species, catbirds, like several other species, do not undergo these biochemical changes.

Potential mechanistic regulators of the observed phenotypic changes in avian migrants include the NR class of transcription factors. Recent studies have suggested that lipid-activated PPARs may mediate muscle metabolic changes via direct regulation of their target genes involved in β-oxidation (Corder et al., 2016; Dick, 2017; Guglielmo et al., 2002; Meng et al., 2005). Indeed, the three PPAR isoforms recently cloned from catbirds share high sequence homology and similar functional properties with their mammalian homologs, including transcriptional activity and regulation of lipid uptake and oxidation in myocytes (Hamilton et al., 2018). To determine how these genes are associated with one another in free-living songbirds, we used a network analysis to visualize these associations in the context of their functional roles (Fig. 4). Genes that share functional roles (indicated by the shape of the nodes) were significantly positively correlated. The nuclear receptors and coactivators correlate most strongly with each other and oxidative metabolic genes (*CS* and *MCAD*) and a fat storage gene (*Plin3*), but not with genes involved in fat transport (*CD36*, *FABPpm*, *FATP1*) and catabolism (*LPL*, *HSL*, *ATGL*). NRs and fat utilization genes may be indirectly linked through the expression of oxidative genes such as *CS*. FATP1, GOT2 and Plin3 were positively correlated with CS, suggesting that a high oxidative metabolism may indirectly increase the expression of fat utilization proteins. We investigated the potential involvement of NRs by characterizing the regulation of PPARs, ERRs and their target genes in the pectoralis muscle and the heart. The expression of fatty acid transporters (CD36, FATP1,

Table 4. Gray catbird PPAR target gene expression in the pectoralis in Oxford, Ohio, USA, during fall pre-migration, fall migration, spring migration and breeding (2012–2014), and in Indian Church Village, Belize, during tropical overwintering (2014)

Gene	Pre-migration	Fall migration	Tropical overwintering	Spring migration	Breeding	*P*
Sample size	10	9	7	10	8	
CD36	1.00±0.11	1.02±0.10	1.02±0.06	0.75±0.06	0.73±0.09	0.03
ATGL	1.00±0.13^{a,b}	1.12±0.13^{a,b}	0.59±0.09^{a}	1.23±0.19^{a,b}	1.38±0.27^{b}	0.05
MCAD	1.00±0.13^{a,b}	1.04±0.09^{a,b}	0.80±0.20^{a}	1.49±0.15^{b}	1.31±0.17^{a,b}	0.02
FABPpm	1.00±0.07^{a,b}	0.94±0.09^{a}	1.32±0.10^{a,b}	1.33±0.11^{b}	1.13±0.11^{a,b}	0.01
FATP1	1.00±0.09	0.93±0.16	1.93±0.47	1.08±0.12	1.03±0.16	0.13
HSL	1.00±0.19^{a,b}	0.72±0.13^{a}	0.95±0.35^{a,b}	1.69±0.24^{b}	0.65±0.13^{a,b}	0.03
LPL	1.00±0.12^{a}	0.93±0.15^{a,b}	0.53±0.03^{b}	0.81±0.07^{a,b}	0.79±0.07^{a,b}	0.02
Plin2	1.00±0.12	1.54±0.17	0.91±0.15	1.36±0.21	0.93±0.10	0.02
Plin3	1.00±0.12^{a,b}	1.41±0.20^{c}	1.49±0.18^{a,c}	1.60±0.32^{a,b,c}	0.96±0.07^{b}	<0.01
CS	1.00±0.10	1.10±0.12	1.73±0.33	1.07±0.10	0.92±0.17	0.06

Data are presented as means±s.e.m. Different letters indicate statistical differences in log-transformed gene expression (*ATGL*, *FABPpm* and *Plin2* not log transformed) among stages determined by one-way ANOVA and *post hoc* Tukey's test or Wilcoxon and Kruskal–Wallis rank sums test and Steel–Dwass pair-wise comparisons.

FABPpm), the fatty acid oxidation enzyme (MCAD) and the tricarboxylic cycle enzyme (CS) did not change throughout the annual cycle in the heart (Table 5). In the pectoralis, the relative expression of PPAR and ERR isoforms did not strictly parallel changes in metabolism and muscle structure in catbirds. Instead, we observed small but significant differences in PPARα and δ, PGC-1α and β, and ERRβ expression in pre-migration and fall and spring migration stages (Table 4, Fig. 5). PPARδ is involved in regulating muscle oxidative capacity and muscle fatty acid oxidation in mammals (Ehrenborg and Krook, 2009). Consistent with it playing a conserved role in enhancing muscle oxidative capacity in preparation for long-distance flight, catbird PPARδ was upregulated in pectoralis muscle during pre-migration. In mammals, PPARα regulates fatty acid transporter and oxidation genes (Bensinger and Tontonoz, 2008). Supporting a similar role in catbirds, the expression of PPARα was significantly upregulated during fall migration, when lipid fuel utilization in the pectoralis is crucial to maximize metabolic performance. ERRβ is involved in regulating metabolism in various tissues (Alaynick, 2008; Huss et al., 2015), and was also upregulated in flight muscle during fall migration. The transcript levels of the nuclear receptor coactivators PGC-1α and PGC-1β were coordinately increased during pre- and fall migration, consistent with activation of PPAR and ERR target gene expression. Preparation for migration and early migration may require a large amount of energy utilization, necessitating increased lipid transport into energy consuming tissues. Consistent with this notion, in pectoral muscle lipoprotein lipase (LPL) expression was highest during pre-migration, and expression of CD36, a fatty acid transporter, was highest during pre- and fall migration.

In the tropics, expression of target genes involved in oxidative and catabolic pathways (*PPARα*, *PGC-1α*, *PGC-1β*, *ERRβ*, *FABPpm*, *Plin3*) were upregulated in flight muscle despite a low metabolic capacity. Genes involved in fatty acid oxidation, *LPL*, *MCAD* and *ATGL*, were significantly downregulated in the tropics, indicating a shift away from fat catabolism, consistent with increased fat stores observed during wintering (Corder et al., 2016). Although our data do not identify the definitive driver of gene expression patterns in the tropics, dietary changes during wintering may be a contributing factor, and are supported by the significant increase in RER observed in the overwintering birds. The natural ligands for NRs in birds are unknown, but differences in dietary lipid species may have important effects on PPAR and ERR activity. Fatty acids strongly activate PPAR pathways in catbirds (Hamilton et al., 2018), and increased PPAR activation is considered to be a potential benefit of consuming polyunsaturated fatty acids (PUFA) in migrants (Guglielmo, 2018). *PPARδ* mRNA abundance was lowest in the flight muscle of yellow-rumped warblers consuming primarily n-3 PUFA compared with birds eating n-6 PUFA and monounsaturated fatty acids during fall migration (Dick, 2017). Collectively, the expression patterns of energy and lipid metabolic genes correlated with periods of increased energy demand associated with migratory flight and during tropical wintering in the pectoralis. Given the relatively modest dynamic in NR and cofactor expression, it is clear that phenotypic plasticity over the annual cycle is not solely mediated by expression levels of these factors.

Conclusions

Organisms commonly undergo fluctuating patterns of stability and change, due to both seasonal and stochastic events, yet full annual cycle research investigating how animal physiology responds to environmental changes is understudied in animal ecology (Marra et al., 2015). The present study, spanning the major life history stages of a migratory bird, helps us to understand the flexibility in

Table 5. Gray catbird PPAR target gene expression in the heart from individuals collected in Oxford, Ohio, USA, during fall pre-migration, fall migration, spring migration and breeding (2012–2014), and in Indian Church Village, Belize, during tropical overwintering (2014)

Gene	Pre-migration	Fall migration	Tropical overwintering	Spring migration	Breeding	*P*
Sample size	11	8	9	10	7	
CD36	1.00±0.10	1.34±0.11	0.95±0.05	1.26±0.11	1.19±0.07	0.15
FATP1	1.00±0.14	0.76±0.12	0.75±0.16	0.95±0.07	0.76±0.07	0.59
FABPpm	1.00±0.09	1.00±0.15	1.05±0.28	1.41±0.20	1.53±0.34	0.29
MCAD	1.00±0.15	0.99±0.10	1.04±0.21	0.95±0.30	1.96±0.59	0.47
CS	1.00±0.09	1.26±0.17	1.26±0.19	1.31±0.19	1.12±0.10	0.54

Data are presented as means±s.e.m. Different letters indicate statistical differences in gene expression (*MCAD* was log transformed) among stages determined by one-way ANOVA and *post hoc* Tukey's test or Wilcoxon and Kruskal–Wallis rank sums test and Steel–Dwass pair-wise comparisons.

and regulation of energy metabolism, and can inform other systems, including migration of other organisms, cold acclimatization and hibernation. This study builds on our previous work (Corder et al., 2016) on the role of PPAR receptors in the regulation of phenotypic flexibility across a migrant bird's annual cycle. However, small mean fold changes in gene expression and challenging interpretation of expression patterns warrant further examination of these pathways. Comparing these data with results from future laboratory studies would likely elucidate the involvement of these pathways (Guglielmo, 2018). Specifically, studies administering PPAR agonists or antagonists, manipulating fatty acid content of the diet, and controlling exercise to induce metabolic phenotypes or non-migratory phenotypes would help discern how intrinsic and extrinsic factors influence PPAR pathways and identify specific roles of PPARs in phenotypically flexible avian systems.

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Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization: K.J.D., D.E.R., J.M.H., P.J.S.; Methodology: J.M.H., P.J.S.; Formal analysis: K.J.D., J.M.H., P.J.S.; Investigation: K.J.D., K.R.C., A.H., P.J.S.; Resources: J.M.H.; Data curation: P.J.S.; Writing - original draft: K.J.D., P.J.S.; Writing - review & editing: K.J.D., K.R.C., A.H., D.E.R., J.M.H., P.J.S.; Visualization: K.J.D.; Supervision: D.E.R., J.M.H., P.J.S.; Project administration: D.E.R., J.M.H., P.J.S.; Funding acquisition: D.E.R., J.M.H., P.J.S.

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Supplementary information
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