Integrative and Comparative Biology

Integrative and Comparative Biology, volume 64, number 6, pp. 1859–1870
https://doi.org/10.1093/icb/icae137

Society for Integrative and Comparative Biology

COMPLIMENTARY/POSTER SESSION PAPER

A Comparison of the Mitochondrial Performance between Migratory and Sedentary Mimid Thrushes

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From the symposium "Recent advances in the mechanistic understanding of avian responses to environmental challenges" presented at the annual meeting of the Society for Integrative and Comparative Biology, January 3–7, 2024.

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Synopsis Birds exhibit a variety of migration strategies. Because sustained flapping flight requires the production of elevated levels of energy compared to typical daily activities, migratory birds are well-documented to have several physiological adaptations to support the energy demands of migration. However, even though mitochondria are the source of ATP that powers flight, the respiratory performance of the mitochondria is almost unstudied in the context of migration. We hypothesized that migratory species would have higher mitochondrial respiratory performance during migration compared to species that do not migrate. To test this hypothesis, we compared variables related to mitochondrial respiratory function between two confamilial bird species—the migratory Gray Catbird (*Dumetella carolinensis*) and the non-migratory Northern Mockingbird (*Mimus polyglottos*). Birds were captured at the same location along the Alabama Gulf Coast, where we assumed that Gray Catbirds were migrants and where resident Northern Mockingbirds live year-round. We found a trend in citrate synthase activity, which suggests that Gray Catbirds have a greater mitochondrial volume in their pectoralis muscle, but we observed no other differences in mitochondrial respiration or complex enzymatic activities between individuals from the migrant vs. the non-migrant species. However, when we assessed the catbirds included in our study using well-established indicators of migratory physiology, birds fell into two groups: a group with physiological parameters indicating a physiology of birds engaged in migration and a group with the physiology of birds not migrating. Thus, our comparison included catbirds that appeared to be outside of migratory condition. When we compared the mitochondrial performance of these three groups, we found that the mitochondrial respiratory capacity of migrating catbirds was very similar to that of Northern Mockingbirds, while the catbirds judged to be not migrating were lowest. One explanation for these observations is these species display very different daily flight behaviors. While the mockingbirds we sampled were not breeding nor migrating, they are highly active birds, living in the open and engaging in flapping flights throughout each day. In contrast, Gray Catbirds live in shrubs and fly infrequently when not migrating. Such differences in baseline energy needs likely confounded our attempt to study adaptations to migration.

Introduction

As part of their annual migrations, birds of many species take off and engage in powered flapping flight for 10s of hours or even multiple days without resting (Gill et al. 2005; Hedenström 2010). Such sustained flapping flight requires among the highest metabolic rates among vertebrates (Schmidt-Nielsen 1972; Norberg 1990; Suarez 1992). The extent to which populations of birds engage in seasonal movements varies from completely resident species, to occasional or short-distance migrations, to the most extreme annual transoceanic movements (Payevsky 2020). Numerous changes in physiology have been associated with long-distance migration; compared to species that do not migrate or migrate shorter distances, longer-distance migrants exhibit higher hematocrit and hemoglobin, greater fuel deposition, and increased exercise endurance (Corman et al. 2014; Yap et al. 2019; Hahn et al. 2022; Elowe et al. 2023).

Advance Access publication August 9, 2024

Long-distance migration is fueled primarily by stored fat, although skeletal muscle has also been shown to be an important fuel for some bird species that migrate long distances (Guglielmo 2018; DeMoranville et al. 2019; Dick and Guglielmo 2019; Elowe et al. 2023). Importantly, catabolism of skeletal muscle during migration produces water, which may aid in osmotic homeostasis (Gerson and Guglielmo 2011). Recently, a study on European Starlings (*Sturnus vulgaris*) found that mitochondrial respiration of the pectoralis muscle is highly correlated with whole-organism basal metabolic rate (Casagrande et al. 2023); thus, we focus on mitochondrial traits in the pectoralis muscle. While many studies have documented the physiological adaptations for migration, few are focused on mitochondria, the primary producer of energy in the form of ATP (Bowlin et al. 2010). ATP is produced within the mitochondria via oxidative phosphorylation (OXPHOS), which occurs along the electron transport system (ETS) (Gnaiger et al. 2018). Measuring mitochondrial respiratory performance is challenging and experts debate the best measures of mitochondrial respiratory function (Brand and Nicholls 2011; Gnaiger 2019). However, there is general agreement that the best measures of mitochondrial function are made with "living" mitochondria immediately after a tissue sample is obtained (Yin and Shen 2022). Moreover, experts agree that precise measurements of oxygen consumption by mitochondria while the electron transport system is chemically manipulated is the foundation for measures of mitochondrial respiratory function (Brand and Nicholls 2011; Gnaiger 2019).

For this study, our goal was to use a liquid-phase respirometer to compare the respiratory physiology of intact mitochondria isolated from the pectoralis muscle of a long-distance migrant to the respiratory physiology of mitochondria isolated from the pectoralis muscle of a closely related non-migrant. To accomplish this, we utilized our mobile laboratory to study mitochondria isolated from birds at the field site where they were captured and euthanized. We focused on two species belonging to the family Mimidae: Gray Catbird (*Dumetella carolinensis*) and Northern Mockingbird (*Mimus polyglottos*). Northern Mockingbirds are non-migratory, remaining in the same small territory throughout the year (Farnsworth et al. 2020). While this species does not migrate, we would expect energy demand to be highest for this species during winter and breeding; thus, we sampled individuals outside of these periods. Previous work has demonstrated mitochondrial variables adjust to meet the demands of winter thermogenesis in resident species (Milbergue et al. 2022). Most Gray Catbirds that breed in eastern North America migrate around the Gulf of Mexico in September and October each year, while others fly directly across the Gulf (Eddins and Rogers 1992). It has also been shown that select Gray Catbirds winter along the Northern Gulf of Mexico, with some staying in Coastal Alabama for the winter (Ryder et al. 2011). To determine if catbirds included in our study were actively migrating or beginning winter residency in the area, we recorded variables (fat score, hematocrit, and right pectoralis mass) commonly used to indicate the migratory status of birds (Marsh 1984; Woodrey and Moore 1997; Krause et al. 2016). By comparing catbirds and mockingbirds captured at the same time and in the same location, we tested the hypothesis that mitochondrial respiratory performance would be higher in a migratory species when in a migratory state than in a closely related non-migrant. Thus, our focus was comparing catbirds when individuals would be expected to be in a high energetic state compared to mockingbirds, which were neither breeding nor migrating.

Methods

Study area and sampling design

Collection took place in October 2020 at the Auburn Gulf Coast Research and Extension Office in Fairhope, AL, USA (coordinates: 30.54345, −87.88599). All experiments and collection took place in accordance with Auburn University Institutional Animal Care and Use Committee (PRN #2020–3791), Migratory Bird Permit #MB42176A-2 and Alabama State Permit #2021035347268680. We chose to compare Gray Catbirds and Northern Mockingbirds because the former is readily found along the Alabama Gulf Coast during migration, while the latter can be found throughout the year. Importantly, these species can vary in body mass, Gray Catbirds exhibit a large range in body mass from 23.2 to 56.5 g (Robert et al. 2020) depending on fat and muscle stores that are deposited prior to migration, while Northern Mockingbirds have a range of 48.3 to 49.7 g along the Gulf Coast (Farnsworth et al. 2020).

Choice of tissue

Previous work has demonstrated that the pectoralis muscle in the Gray Catbird exhibits hypertrophy in preparation for migration, increasing up to 35% in preparation for fall migration (Marsh 1984; DeMoranville et al. 2019). The pectoralis muscle, which is critical for flight, uses 30–40% of a bird's total oxygen consumption while at rest (Casagrande et al. 2023); the oxygen demands of the pectoralis muscle are likely substantially greater while supporting the downstroke during flight. Thus, we selected the pec-

toralis muscle as the source of mitochondria in this investigation.

Approach to measuring mitochondrial performance

We determined mitochondrial respiratory performance using oxidative phosphorylation (OXPHOS) capacity and mitochondrial respiratory control (also known as RCR) in isolated mitochondria. OXPHOS capacity is defined as the maximum ATP production by coupled mitochondria (Koch et al. 2021). To measure this, we use the maximum rate of oxygen utilization, or state 3 respiration, as a proxy for ATP production when the mitochondria are provided unlimited oxygen, substrate, and ADP. Mitochondrial respiratory control provides insight into the relative performance of coupled mitochondria and is determined by dividing state 3 by state 4. State 4 respiration, or basal respiration, is defined as the minimum rate of oxygen utilization by the mitochondria when no ADP is present but substrate and oxygen are still abundant (Brand and Nicholls 2011). State 4 respiration is a proxy for proton leak across the inner mitochondrial membrane. These measurements were completed with different substrates, as detailed in the methods that allowed us to quantify complex I mediated respiration via (palmitoylcarnitine) or independent (pyruvate, malate, and glutamate) of the β-oxidation pathway and complex II respiration (succinate). In addition, we also measured the enzymatic activities of ETS complexes I–IV, as changes in complex activity can provide a mechanism for altering OXPHOS (Bundgaard et al. 2019).

Data collection

We trapped from sunrise to sunset. Birds were trapped using mist nets with the aid of call playback and decoys. Upon initial capture, morphometrics were collected, including fat and muscle scores (scored using a 0–3 scale modified from a 0–4 scale by Salewski et al. 2009), body mass (g), and wing chord (mm). Birds were aged as either after-hatching year (AHY) or hatching year (HY) based on plumage characters (Pyle 1997). Blood was collected via venipuncture of the brachial vein using a 26-gauge needle. The blood was collected in 75-µl microhematocrit capillary tubes, following standard procedures (Sheldon et al. 2008). The blood was centrifuged for 10 min at 17,700 g to measure hematocrit (%). Hematocrit was measured based on the length of erythrocytes divided by the length of whole blood plasma within the tube. Ketone, β-hydroxybutyrate (BOH) was also measured during this step following procedures previously outlined by Lindholm et al. (2019) using the point of care device ketone meter (FreeStyle Precision Neo, Abbott, IL, USA). Around 1.5 µl of blood was placed on a test strip and the value provided was recorded. Birds were euthanized via decapitation in accordance with IACUC PRN#2020-3791 and following humane guidelines as deemed by the AVMA Guidelines for the Euthanasia of Animals: 2020 Edition (Underwood and Anthony 2020). Sex was determined via internal examination of the gonads, where the presence of testes indicated male and an ovary indicated female. We collected a total of 11 Gray Catbirds and 10 Northern Mockingbirds.

Next, the right pectoralis was excised, weighed, and divided for mitochondrial isolation. The remaining tissue was flash-frozen in liquid nitrogen and later transferred to a −80°C freezer for future analysis. For all plate assays, samples were selected at random on 96-well plates. We maintained an inter-assay coefficient of variation (CV) of ≤15% and intraassay ≤10%.

Mitochondrial isolation and respiration

Mitochondrial isolation was conducted following procedures previously outlined (Zhang et al. 2018). A 1–2 g sample of the right pectoralis was put into a skeletal muscle isolation solution pH of 7.5 (100 mM KCl, 40 mM Tris-HCl, 10 mM Tris Base, 1 mM $MgCl^2$, 1 mM EGTA, 0.2 mM ATP, and a 0.15% BSA solution) at a 1:10 ratio and then minced with scissors. After mincing, it was homogenized for 5 s with a VITRIS electric homogenizer at half power. Digestion was then accomplished by adding a protease from *Bacillus lincheniformis*, made fresh using the isolation solution with BSA at 5 mg per gram of wet muscle. The homogenate was then mixed for 7 min by swirling vigorously every 30 s. Termination of digestion was accomplished by adding an equal volume of the original isolation solution. The homogenate was centrifuged at 500 g for 10 min at 4°C. The supernatant was decanted using cheesecloth and the pellet centrifuged at 4500 g for 15 min at 4°C. The pellet was then resuspended with an equal volume of original BSA solution using a rubber policeman for a final spin at 4500 g for 15 min. Lastly, the supernatant was discarded once again, and the pellet resuspended in isolation solution but without the 0.15% BSA solution and centrifuged at 3500 g for 10 min at 4°C. The supernatant was removed, and the final mitochondrial pellet was resuspended in a 0.25–0.75 ml range of a mannitol-sucrose solution depending on the final pellet size. The sample was transferred to a Dounce homogenizer and resuspended with 4–5 passes.

Mitochondrial respiratory states were quantified polarographically in a respiration chamber maintained at 40°C (Oxytherm; Hansatech Instruments, UK) (Messer et al. 2004). We quantified mitochondrial states as indi-

cators of mitochondrial efficiency. We incubated 20 ul of the isolated mitochondria in respiration buffer (pH 7.0) with 220 mM mannitol, 70 mM sucrose, 10 mM Tris-HCL, and 1 mM EGTA at 40°C. The respiration buffer amount was adjusted for the total volume in the chamber to be 1 ml. Complex I respiration was tested using 2 mM pyruvate, 2 mM malate, and 10 mM glutamate as substrates. We also tested complex I respiration using 4 mM of the substrate palmitoylcarnitine; complex II was tested using 5 mM succinate with 10 uM rotenone to inhibit complex I.

State 3 respiration was initiated by adding 0.25 mM ADP to the mitochondria and respiratory substrates in the chambers. State 3 was determined as the highest rate ADP is converted to ATP when ADP is added with excess substrate and determined using oxygen use as a proxy and provides an indicator of the maximum rate of oxygen utilization by the mitochondria, hereafter OXPHOS capacity (Brand and Nicholls 2011; Zhang et al. 2018). Oligomycin was added to calculate oligomycin-induced state 4 (state 4o), and this is the state 4 we report in our results, which prevents contamination of ATP recycling molecules such as ATPases (Racker 1963). Mitochondrial respiratory control (RCR) was calculated by dividing state 3 by state 4 with oligomycin (Zhang et al. 2018). Respiration rates were normalized to total mitochondrial protein concentration using the Bradford assay technique.

Enzymatic assays

Citrate synthase activity assays, used as a proxy for mitochondrial volume, were conducted on frozen pectoralis homogenate samples following methods previously reported (Andersen et al. 2003; Larsen et al. 2012). To make the homogenate, a volume of 750 µl of lysis buffer was added to 30–50 mg of tissue, homogenized, and spun at 1500 g for 15 min at 4°C. The supernatant was then collected, and the Bradford assay technique was used to determine protein content. Citrate synthase activity was measured spectrographically at 40°C as an increase in absorbance from 5,5′-dithiobis-2-nitrobenzoic acid reduction over a minute, with 10-s intervals. Calculations of enzyme activity followed Spinazzi et al. (2012).

Electron transport system complex activities were determined using the frozen isolated mitochondria samples. ETS complexes I, III, and IV were determined using methodology from Spinazzi et al. (2012) with minor modifications. Complex II activity was determined as described by Kavazis et al. (2009). All activities were determined spectrophometrically. Frozen isolated mitochondria samples were subjected to three freezing and thawing cycles to lyse membranes before analysis.

Complex I (NADH: ubiquinone oxidoreductase) was measured as the decrease in absorbance from NADH oxidation by decylubiquinone minus rotenone resistance activities. Complex II (succinate dehydrogenase) was measured as the decrease in absorbance from 2,6-dichloroindophenol reduction. Complex III (decylubiquinol cytochrome *c* oxidoreductase) was measured as the increase in absorbance from cytochrome *c* reduction minus antimycin A resistant activity. Complex IV (cytochrome *c* oxidase) was measured as the decrease in absorbance from cytochrome *c* oxidation minus KCN-resistant activity. Complex activities were standardized to total protein content using the Bradford assay technique. Citrate synthase and complex activities were measured in triplicate with the mean reported. We maintained an inter-assay coefficient of variation (CV) of ≤15% and intraassay of ≤10%.

Data analyses

All statistical tests were completed using R version 4.2.3 (R Core Team 2023) and RStudio version 2023.6.1.524 using the linear model function (Posit Team 2023). Using methods outlined in Zuur et al. (2010), we tested the assumptions of our linear models and for outliers (Zuur et al. 2010). This identified one clear outlier for Complex IV, which was removed. All dependent variables were normally distributed, so we used the linear model function for our analyses. For BOH, two Gray Catbird individuals reached the maximum value (10 mM) that the point of care device measured. For these, we assigned them the value 10 but note that it may have been higher than this value. Since the body mass was higher in the non-migrants than migrants and since it is known that body mass impacts mitochondrial respiration (Park et al. 2020; Boël et al. 2023), we included body mass as a covariate in all models. We did not include sex in the model since we had a small sample size, and because male and female migrants travel similarly during migration, sex-specific effects are expected to be limited. For age, the migrants were mainly HY birds and the non-migrants AHY. Thus, we removed the AHY ($n = 2$) birds from the catbirds and the HY birds from the mockingbirds ($n = 1$) to test if this had an overall impact of the results. It did not impact the results overall (see Supplementary Information for details).

To evaluate differences between migratory and non-migratory birds, we compared body mass, relative pectoralis mass (which was calculated by dividing the right pectoralis mass by body mass), hematocrit, BOH, citrate synthase activity, state 3 and state 4 respiration, RCR, and complexes I–IV activities between species, including body mass as a covariate for the respiration states and RCR.

Table 1 Physiological metrics, descriptive stats and results comparing catbirds and mockingbirds.

Parameters	Catbird mean ± standard deviation (std) (*n*)	Mockingbird mean ± std (*n*)	Estimate (β)	*F*-statistic	*P*-value
Body mass (g)	39.2 ± 4.8 (11)	48.89 ± 5.01 (10)	9.68	20.6	<0.001
Pectoralis (% body mass)	5% ± 0.6% (11)	7% ± 0.3% (10)	0.02	52.9	<0.001
Hematocrit (%)	51% ± 5% (8)	51% ± 2% (9)	0.01	0.13	0.72
BOH (mmol/L)	5.33 ± 2.63 (11)	6.11 ± 2.23 (10)	0.78	0.54	0.47

Since the results for several variables for Gray Catbirds were widely variable and to determine if individuals should be assigned to different phenotypes, we conducted a principal component analysis (PCA), including all catbirds and mockingbirds in the model, and included variables commonly used to indicate migratory state in-hand, including hematocrit, fat score, and relative pectoralis mass. While ornithologists typically use muscle score to evaluate pectoralis size in hand, we used the non-subjective and quantitative measurement of relative muscle mass in this analysis, although it should be noted that this measure does not take fat score into account.

PCA was conducted as previously reported (Corman et al. 2014; Josefson and Hood 2023). We used the "prcomp" function from the R-package "stats." For missing data from variables, we used the mean imputation method for PCA (Podani et al. 2021). Data were converted to numeric and then scaled and centered to ensure the variables had equal weight prior to analysis. We used a scree plot to determine the number of PCs to retain and the elbow method to determine our ideal number of clusters (Rolshausen et al. 2013). The loadings were calculated for the components and were considered significant if they explained a large amount of the variance and had an eigenvalue of >1. After PCA analysis, we grouped individuals based on the results and tested the normalcy of the data once again. Since this data was normally distributed, we repeated the linear model analysis on the mitochondrial variables using three groups: mockingbirds, catbirds that displayed the migrating phenotype, and catbirds that displayed a not migrating phenotype, as described below.

Results

All mockingbirds had fat scores of 0 and muscle scores of 0 ($n = 2$) or 1 ($n = 8$). Catbirds had variable fat scores from 0 to 3 and muscle scores from 0 to 2. For hematocrit and BOH, we found no significant differences between species. However, both relative pectoralis mass and body mass were significantly higher in mockingbirds compared to catbirds ($P < 0.001$; Table 1). For citrate synthase, there was a trend suggesting that catbirds have a higher mitochondrial volume in their pectoralis than mockingbirds ($P = 0.07$; Fig. 1). For our mitochondrial respiration results, no significant differences were found between catbirds and mockingbirds with respiration using all three substrate/substrate cocktails (Table 2). We did not find any significant differences between the complex enzymatic assays, including complexes I–IV (Table 2).

The PCA analyses revealed that PC1 and PC2 explained 74% of the variation in the data (Table 3). For PC1, fat score and relative pectoralis mass explained most of the variation, although pectoralis had a negative relationship to fat score. For PC2, % HCT explained most of the variation, followed by the pectoralis (Table 3). Cluster visualization (Fig. 2), demonstrates that a subset of catbirds ($n = 5$) cluster separately from the other catbirds and mockingbirds. We then examined the physiological parameters of these five catbirds and concluded that they appeared to match a migrant phenotype with a relatively high fat score and high hematocrit (Table 4). The other catbirds grouped with the mockingbirds matched a non-migratory phenotype with low body fat and low hematocrit. We then repeated the analysis for our mitochondrial variables using the three groups: catbirds in cluster 1 (migrating phenotype), catbirds in cluster 2 (not migrating phenotype), and mockingbirds (Table 5). While most vari-

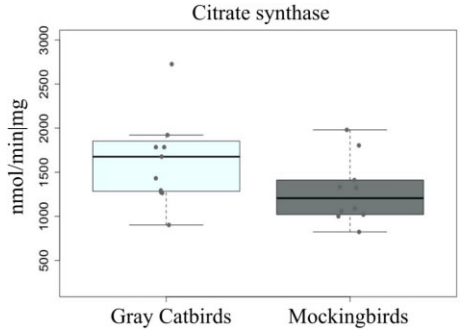

Fig. 1 Mitochondrial volume as indicated by citrate synthase activity in Gray Catbirds and Northern Mockingbirds. A linear model indicates that variation between groups approached significance ($P = 0.07$).

Table 2 Results and statistics for mitochondrial variables testing for species effect.

	Catbird mean ± std (*n*)	Mockingbird mean ± std (*n*)	Estimate (β)	*F*-statistic	*P*-value
Mitochondrial volume					
Citrate synthase (nmol/min/mg protein)	1761 ± 702 (11)	1284 ± 369 (10)	−477	3.68	0.07
Mitochondria respiration with pyruvate, malate, and glutamate substrates					
State 3 (nmol O_2/mg protein/min)	491.8 ± 158.1 (11)	513.1 ± 147.3 (10)	29.1	0.05	0.77
Body mass			−0.810		0.92
State 4 (nmol O_2/mg protein/min)	29.11 ± 7.22 (11)	28.59 ± 7.79 (10)	−2.14	0.12	0.66
Body mass			0.170		0.64
RCR	16.74 ± 2.14 (11)	18.30 ± 3.77 (10)	2.87	1.04	0.17
Body mass			−0.140		0.38
Mitochondria respiration with palmitoylcarnitine substrate					
State 3 (nmol O_2/mg protein/min)	283.0 ± 83.3 (11)	327.8 ± 125.8 (10)	81.7	0.77	0.23
Body mass			−3.81		0.45
State 4 (nmol O_2/mg protein/min)	34.59 ± 9.70 (11)	36.43 ± 10.87 (10)	3.85	0.18	0.56
Body mass			−0.210		0.67
RCR	8.63 ± 3.57 (11)	9.47 ± 3.51 (10)	1.090	0.16	0.63
Body mass			0.0300		0.88
Mitochondria respiration with succinate substrate					
State 3 (nmol O_2/mg protein/min)	377.8 ± 79.3 (11)	419.0 ± 106.3 (10)	57.2	0.57	0.35
Body mass			−1.66		0.71
State 4 (nmol O_2/mg protein/min)	94.24 ± 19.53 (11)	105.1 ± 19.2 (10)	8.53	0.86	0.49
Body mass			0.240		0.79
RCR	4.01 ± 0.37 (11)	3.96 ± 0.60 (10)	0.200	0.71	0.51
Body mass			−0.0200		0.26
Complex enzymatic activity					
Complex I (nmol/min/mg protein)	302.4 ± 162.9 (10)	274.3 ± 136.3 (10)	−28.1	0.17	0.68
Complex II (nmol/min/mg protein)	309.9 ± 781.2 (9)	869.2 ± 510.3 (7)	88.0	0.18	0.68
Complex III (nmol/min/mg protein)	401.5 ± 185.2 (9)	382.4 ± 154.5 (10)	−19.1	0.06	0.81
Complex IV (nmol/min/mg protein)	331.7 ± 167.6 (9)	315.6 ± 132.1 (10)	16.2	0.06	0.82

∗For mitochondrial respiration, we included body mass as a covariate. States 3, 4, and RCR were tested using three substrate combinations. Gray Catbird is the reference group for the linear model results.

Table 3 Principal components 1 and 2 results.

	PC1	PC2
Overall:		
Percent variance	0.48	0.26
Eigenvalue	2.40	1.29
Individual variable weights:		
Fat score	0.69	-0.08
Hematocrit (%)	0.34	0.91
Pectoralis (% body mass)	−0.64	0.40

ables were similar between groups, a trend suggested that RCR with pyruvate, malate, and glutamate substrate displayed a trend toward being highest in mockingbirds, followed by the migrating catbird, and lastly, the not migrating catbird ($P = 0.07$; Fig. 3). Interestingly, although not significant, the not migratory catbird phenotype trended higher for citrate synthase than the migratory phenotype (Table 5).

Discussion

We assessed the mitochondrial respiratory performance of Northern Mockingbirds, which do not migrate, and Gray Catbirds, which engage in long-distance migration. Previous studies of other species of songbirds have shown that migrants have an increased aerobic performance compared to non-migrants (Krause et al. 2016). Moreover, a recent study on populations of White-crowned Sparrows (*Zonotrichia leucophrys*) that migrate show seasonally elevated RCR, state 3, and citrate synthase compared to conspecific populations that do not migrate (Rhodes et al. 2024). Thus, we pre-

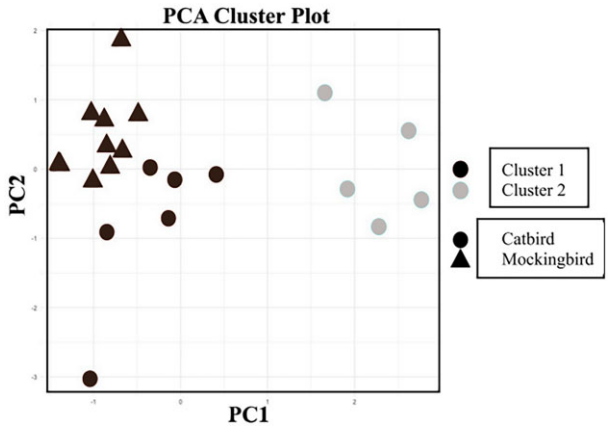

Fig. 2 PCA cluster analysis results. PC1 is weighted for fat score (+) and relative pectoralis mass (−) and PC2 is weighted for hematocrit.

dicted that we would observe higher OXPHOS capacity for mitochondria isolated from the pectoralis muscle of migratory Gray Catbirds vs. non-migratory Northern Mockingbirds. Contrary to this prediction, we found no significant differences between the respiration rates or the ETS complex activities of the Northern Mockingbirds and Gray Catbirds that we sampled on the Alabama Gulf Coast. However, there was a trend suggesting the Gray Catbirds have a greater mitochondrial volume (measured by the proxy citrate synthase) in their pectoralis muscles than Northern Mockingbirds.

Previous work on Gray Catbirds has shown that pectoralis mass and whole-animal summit metabolic rate are higher in individuals in a migratory state vs. individuals on their wintering (non-breeding) grounds, but interestingly, mitochondrial volume, as indicated by citrate synthase was not (DeMoranville et al. 2019). DeMoranville et al. (2019) collected migratory Gray Catbirds during breeding and migratory periods in Ohio and on their wintering grounds in Belize. Given that species and individuals may differ in how they upregulate ATP production (Hood 2024), possibly with some individuals increasing muscle mass with the same mitochondrial volume and others increasing mitochondrial volume and not muscle mass, it is possible that the catbirds in our study displayed a different strategy to meet the energy demands of migration relative to those in DeMoranville et al.'s (2019) study. However, this is unconfirmed since our sampling was limited to one timepoint. Nevertheless, the high variance in common markers of migration status in the catbirds made us question whether the birds we collected represent our targeted, migratory/non-migratory dichotomy.

In designing our study, we chose a study area where Northern Mockingbirds are resident but Gray Catbirds do not breed (Alabama Breeding Bird Atlas 2009). The period in which we collected birds was during the later portion of fall migration for Gray Catbirds along the Alabama Gulf Coast (Robert et al. 2020), and we assumed that the Gray Catbirds that we captured would be actively migrating through the area, exhibiting the typical migratory physiology. This assumption appears to have been incorrect. To investigate the potential migration status of the Gray Catbirds that we caught, we calculated the relative mass of the pectoralis muscle, assessed the amount of fat stored in the abdomen and furcular region, and measured the volume of packed red blood cells in blood samples. For each of these parameters, individuals that are migrating show distinct patterns relative to conspecifics that appear not to be migrating, at least based on their physiological state. The Gray Catbirds that we sampled fell into two distinct groups: one with parameters expected for individuals engaged in active migration and one with parameters expected for individuals that were not migrating. Interestingly, the "not migrating" phenotype catbirds also display relatively low pectoralis mass, which is typically hyper-

Table 4 Phenotypic differences between the catbird groups and mockingbirds determined by PCA analysis.

	Catbird-migrating mean ± std	Catbird-not migrating mean ± std	Mockingbird mean ± std
Included in the PCA:			
Fat score	2.0 ± 0.7	0.2 ± 0.4	0.0 ± 0.0
Hematocrit (%)	53.6% ± 2.7%	45.3% ± 4.7%	51.2% ± 2.3%
Pectoralis (% body mass)	4.88% ± 0.29%	5.87% ± 0.31%	6.95% ± 0.32%
Other phenotypic variables:			
Body mass (g)	43.3 ± 3.8	35.8 ± 1.8	48.8 ± 5.0
Pectoralis mass (g)	2.11 ± 0.21	2.11 ± 0.19	3.41 ± 0.45
Pectoralis score	1.0 ± 0.0	0.67 ± 0.82	0.8 ± 0.4
Age (HY:AHY)	5:0	4:2	1:9
Sex (M:F)	5:0	4:2	3:6 (1 NA)

∗Age includes hatch-year (HY) and after-hatch-year birds (AHY). Sex was missed on one of the mockingbirds (NA).

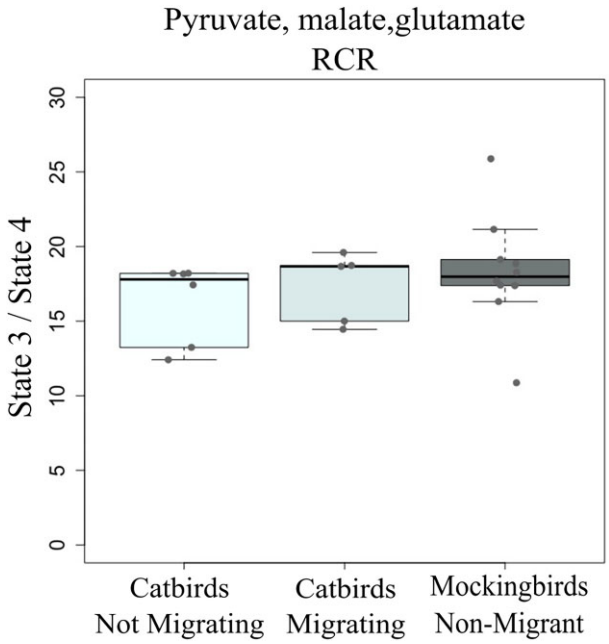

Fig. 3 Mitochondrial respiratory control (RCR) with complex I substrates (pyruvate, malate, and glutamate) for Northern Mockingbirds and the two Gray Catbird phenotypes indicated by PCA. Results are based on a linear model with catbirds divided into migrating and not migrating groups.

trophied during migration in many songbirds (Marsh 1984; Gaunt et al. 1990). Given that muscle catabolism appears to be common during migration (Gaunt et al. 1990; Elowe et al. 2023), this observation provides further support that one of our catbird groups had already completed part of their migratory journey while the other was in the process of migrating. We concluded that, among the catbirds included in our study, we likely sampled both passage migrants that we caught during their migration through the area and birds that were not migrating because they had completed their migration to the Gulf Coast days or weeks prior to capture. This latter group having returned to a non-migratory physiological state.

Another confounding factor in our study was that the group of Gray Catbirds that we judged to be migrating was biased toward smaller hatching-year females. Generally, at least for spring migration, adult catbirds migrate before yearling birds, and male catbirds, on average, migrate before females (Woodrey and Moore 1997). Thus, the bias toward young females in our sample of migrating birds likely reflects that our study dates fell in the last weeks of fall catbird migration.

When we repeated our analysis of mitochondrial performance with three rather than two groups—"migrating" catbirds, "not migrating" catbirds, and mockingbirds—we found an interesting, if unexpected, pattern. Mockingbirds had the highest RCR values—the highest mitochondrial performance values—among any of the groups, but mockingbirds and the "migrating" catbird group had very similar mean RCR values. In contrast, catbirds with physiological markers indicating that they were not actively migrating showed substantially lower, and nearly significantly lower, mean RCR values than migrating catbirds or mockingbirds. If our sampling did in fact include not migratory catbirds, our results would align with recent studies on mitochondrial respiration in migratory birds, which demonstrated that, like many migratory traits, the increased mitochondrial respiration is rapidly reversible (Coulson et al. 2024; Rhodes et al. 2024). Our hypothesis that individuals from migratory species would show increased mitochondrial performance relative to individuals from non-migratory species is clearly falsified. The mitochondrial performance of resident mockingbirds was never lower than migratory catbirds regardless of circumstances.

The sort of comparative study in which we engaged rests on a critical assumption: the only important difference between the populations of birds being compared is the parameter of interest. Unfortunately, even though we chose two bird species from family Mimidae that have many similarities, differences between these birds other than migration behavior seem to have had a large effect on the mitochondrial performance. Upon reflection, we propose that the largest confounding effect in our comparison is the very different daily activity patterns of Northern Mockingbirds and Gray Catbirds. Northern Mockingbirds are notoriously active birds. We propose that the high values for mitochondrial performance that we measured for Northern Mockingbirds are shaped by the demands of the highly active behavioral patterns. They spend the day flying between exposed perches and singing (Farnsworth et al. 2020). Thus, their total time and energy devoted to flapping flight for a day may approach that of a migrating bird. In contrast, when they are not migrating, Gray Catbirds spend their days in dense shrub-scrub habitats where they are relatively much more sedentary and engage in much less frequent flapping flight than mockingbirds (Robert et al. 2020). Additionally, it is unknown how mitochondrial respiration of resident species such as the Northern Mockingbird varies throughout the year depending on energetic demands. Thus, it would be helpful for future work to sample resident species throughout the year, such as during the wintering and breeding periods. In the future, differences in behavior, life history, and differences in daily energy expenditure at the time of comparison would be valuable in migrant and non-migrant studies.

In summary, while Gray Catbirds trend toward having greater mitochondrial volume than Northern Mockingbirds, catbirds displaying a migratory phe-

Table 5 Linear model results of mitochondrial traits of interest using the three groups (mockingbirds, catbirds migrating, and catbirds not migrating) based on the PCA results.

		Estimate (β)	*F*-statistic	*P*-value
Mitochondria respiration with pyruvate, malate, and glutamate substrates				
State 3	Catbirds migrating* vs. not migrating	64.9	0.13	0.60
(nmol O_2/mg protein/min)	Mockingbirds* vs. catbirds migrating	−87.0		0.57
	Mockingbirds* vs. catbirds not migrating	−22.1		0.83
	Body mass	−3.73		0.70
State 4	Catbirds migrating vs. not migrating	−1.69	0.11	0.77
(nmol O_2/mg protein/min)	Mockingbirds vs. catbirds migrating	3.64		0.61
	Mockingbirds vs. catbirds not migrating	1.95		0.70
	Body mass	0.240		0.59
RCR	Catbirds migrating vs. not migrating	3.070	1.31	0.20
	Mockingbirds vs. catbirds migrating	−5.60		0.07
	Mockingbirds vs. catbirds not migrating	−2.53		0.22
	Body mass	−0.270		0.15
Mitochondria respiration with palmitoylcarnitine substrate				
State 3	Catbirds migrating vs. not migrating	−31.3	0.54	0.70
(nmol O_2/mg protein/min)	Mockingbirds vs. catbirds migrating	−53.8		0.59
	Mockingbirds vs. catbirds not migrating	−85.1		0.23
	Body mass	−2.40		0.71
State 4	Catbirds migrating vs. not migrating	−4.82	0.24	0.54
(nmol O_2/mg protein/min)	Mockingbirds vs. catbirds migrating	0.450		0.96
	Mockingbirds vs. catbirds not migrating	−4.37		0.52
	Body mass	0.0100		0.99
RCR	Catbirds migrating vs. not migrating	0.900	0.14	0.74
	Mockingbirds vs. catbirds migrating	−1.89		0.58
	Mockingbirds vs. catbirds not migrating	−0.990		0.67
	Body mass	−0.070		0.75
Mitochondria respiration with succinate substrate				
State 3	Catbirds migrating vs. not migrating	32.7	0.43	0.65
(nmol O_2/mg protein/min)	Mockingbirds vs. catbirds migrating	−86.4		0.34
	Mockingbirds vs. catbirds not migrating	−53.7		0.39
	Body mass	−3.13		0.58
State 4	Catbirds migrating vs. not migrating	3.58	0.56	0.81
(nmol O_2/mg protein/min)	Mockingbirds vs. catbirds migrating	−11.7		0.53
	Mockingbirds vs. catbirds not migrating	−8.14		0.53
	Body mass	0.0800		0.94
RCR	Catbirds migrating vs. not migrating	0.190	0.54	0.62
	Mockingbirds vs. catbirds migrating	−0.370		0.42
	Mockingbirds vs. catbirds not migrating	−0.180		0.57
	Body mass	−0.0400		0.24
Complex enzymatic activity				
Complex I	Catbirds migrating vs. not migrating	−110	0.78	0.26
(nmol/min/mg protein)	Mockingbirds vs. catbirds migrating	83.2		0.32
	Mockingbirds vs. catbirds not migrating	−27.02		0.74

Table 5 Continued

		Estimate (β)	*F*-statistic	*P*-value
Complex II	Catbirds migrating vs. not migrating	170.7	0.27	0.55
(nmol/min/mg protein)	Mockingbirds vs. catbirds migrating	−182.8		0.50
	Mockingbirds vs. catbirds not migrating	−12.1		0.96
Complex III	Catbirds migrating vs. not migrating	83.8	0.29	0.48
(nmol/min/mg protein)	Mockingbirds vs. catbirds migrating	−18.2		0.85
	Mockingbirds vs. catbirds not migrating	65.7		0.53
Complex IV	Catbirds migrating vs. not migrating	−146.4	1.17	0.15
(nmol/min/mg protein)	Mockingbirds vs. catbirds migrating	97.5		0.27
	Mockingbirds vs. catbirds not migrating	−48.9		0.55
Citrate synthase (nmol/min/mg protein)	Catbirds migrating vs. not migrating	−104.9	1.80	0.77

∗The Gray Catbirds were separated into migrating and not migrating groups based on differences in phenotype. Mockingbirds were also included in the analyses. An asterisk (∗) in the table denotes reference group (same throughout).

notype do not have greater respiratory performance than the resident mockingbirds. Additionally, when we tested for differences only between the not migrating catbirds and mockingbirds, we still did not find any differences between the two groups. More detailed research, targeting individuals with known migratory status, will be necessary to determine if Gray Catbirds display OXPHOS adaptations to support migration. This study highlights the need for careful selection of study species in studies of bioenergetic adaptations to migration or other demanding activities. In particular, it is necessary to compare animals with similar activity patterns outside of the variable of interest to detect species-specific effects. Lastly, it should be considered that a caveat to our study is that by isolating mitochondria the cellular context for OXPHOS is lost. No current method of measuring mitochondrial respiration, whether completed in isolated mitochondria, permeabilized cells, or cell culture, will perfectly mimic mitochondrial function *in vivo* (Brand and Nicholls 2011). We recommend that all investigators carefully consider the appropriate method for similar investigations.

Acknowledgments

Special thanks to Auburn Gulf Coast Research and Extension Center (Fairhope, AL, USA) for land access and to Valentine Dudley for field assistance. Special thanks to the symposium organizers of "Recent advances in the mechanistic understanding of avian responses to environmental challenges," Alexander Gerson, Cory Elowe, and Maria Stager at the 2024 Society for Integrative and Comparative Biology Symposium and for the invite to contribute to this issue of ICB. We also thank the two anonymous reviewers for constructive feedback and to the ICB managing editor, Suzanne Miller, for their time.

Funding

This work was supported by the Auburn University Presidential Award for Interdisciplinary Research to W.R.H. and G.E.H. and National Science Foundation grants (Hood OIA1736150, IOS2223528, and Hill IOS2037741). E.M.R. was supported by a Presidential Graduate Research Fellowship from the Auburn Graduate School, the College of Sciences and Mathematics at Auburn University, the National Science Foundation (DGE-1937964), and the Society for the Study of Evolution (R.C. Lewontin Early Award).

Supplementary data

Supplementary data available at *ICB* online.

Conflict of interest

The authors have no conflicts to report.

Data availability

All data is available in the article and in the online supplementary material of this article.

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