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Avian red blood cell mitochondria produce more heat in winter than in autumn

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Abstract

Endotherms in cold regions improve heat-producing capacity when preparing for winter. We know comparatively little about how this change is fueled by seasonal adaptation in cellular respiration. Thus, we studied the changes of mitochondrial function in red blood cells in sympatric Coal (*Periparus ater*), Blue (*Cyanistes caeruleus*), and Great (*Parus major*) tits between autumn and winter. These species differ more than twofold in body mass and in several aspects of their foraging ecology and social dominance, which could require differential seasonal adaptation of energy expenditure. Coal and Great tits in particular upregulated the mitochondrial respiration rate and mitochondrial volume in winter. This was not directed toward ATP synthesis, instead reflecting increased uncoupling of electron transport from ATP production. Because uncoupling is exothermic, this increased heat-producing capacity at the sub-cellular level in winter. This previously unexplored the route of thermogenesis in birds should be addressed in future work.

KEYWORDS

cellular metabolism, erythrocyte, overwintering, oxygen consumption, thermal biology

1 | INTRODUCTION

Endothermic animals that reside year-round in the temperate zone must accommodate pronounced seasonal changes to ambient temperature, photoperiod, precipitation, and food availability. The stresses imposed by these environmental factors intensify in winter, when low ambient temperature increases the energy cost of staying warm at the

Abbreviations: AA, Arachidonic acid; AIC, Akaike information criterion; ATP, Adenosine triphosphate; BAT, Brown adipose tissue (brown fat); CoA, Coenzyme A; CS, Citrate synthase; DHA, Docosahexaenoic acid; EDTA, Ethylenediaminetetraacetic acid; EGTA, Egtazic acid; EPA, Eicosapentaenoic acid; ETS, Electron transport system; here in the context of its maximum respiration capacity when uncoupled from ATP synthase; FCCP, Cyanide-p-trifluoro-methoxyphenyl-hydrazone; FCR, Flow control ratio; that is, a ratio between two different mitochondrial respiration states; HEPES, Hydroxyethyl piperazineethanesulfonic acid; LEAK, Mitochondrial respiration to offset proton leak across the inner mitochondrial membrane when no ATP is being produced; MiR05, Mitochondrial respiration medium 05; OXPHOS, Mitochondrial respiration driving oxidative phosphorylation during ATP synthesis; PMSF, Phenylmethylsulfonylfluoride; RBC, Red blood cell; ROS, Reactive oxygen species; ROUTINE, Baseline mitochondrial respiration with endogenous substrates.

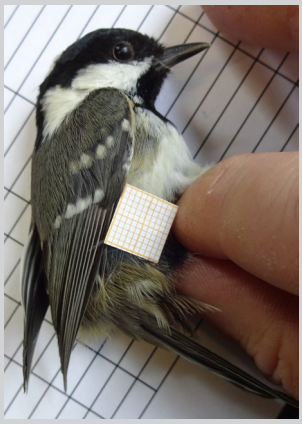
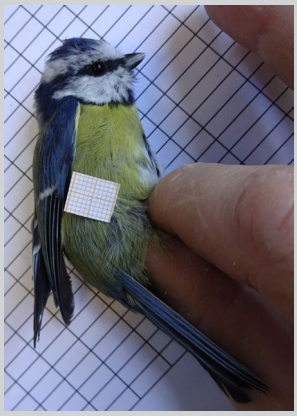
same time as short day length and reduced food availability complicates energy acquisition.[1] Animals meet these environmental challenges by seasonal changes to insulation and body fuel reserves, and appropriate physiological responses to increase heat production or decrease heat loss as needed.[2,3] The resultant winter-adapted phenotypes fall on a continuum of scales from well-insulated, large, and starvation-tolerant animals,[4,5] to smaller animals with improved heat-producing capacity that must maintain high food intake throughout winter on account of a higher surface-area-to-volume ratio and limited fat-storing capacity.[6,7] The ultimate and proximate nature of the behavioral, morphological, and physiological changes that mediate seasonal adaptation are reasonably well understood at organismal and tissue levels, and include hypertrophy of thermogenic tissues and metabolically active organs such as the liver (for reviews, see 7-9). By comparison, considerably less effort has been put into understanding seasonal adaptation of the cellular machinery that fuels aerobic work in tissues when the animal deals with low winter temperature.

In animals, more than 90% of the energy used by working cells is in the form of adenosine triphosphate (ATP) primarily produced by mitochondrial respiration.[10] There is variation in this process depending on the needs of the animal: mitochondrial respiration rate and volume in key metabolic tissues are often upregulated when energy demands increase, such as in winter, at high altitude, or in otherwise cold-adapted animals.[11-14] However, studies that measure changes to mitochondrial respiration rate alone miss the many possible functional changes in mitochondria that animals may undergo to better deal with environmental fluctuations. The oxidative phosphorylation required for ATP production is dependent on the presence of a proton gradient across the inner mitochondrial membrane; this gradient is generated by the mitochondria's electron transport system (ETS), which consumes oxygen in the process.[15] Leakage of protons across that membrane uncouples the link between oxygen consumption and ATP synthesis (so reducing the efficiency of energy production), but generates heat and also reduces the rate at which Reactive Oxygen Species (ROS) are produced.[15] The relative degree of coupling of the ETS to ATP production may thus depend on the animal's needs. For example, energy-limited animals may reduce the proton leak so that ATP production is maintained despite lower overall substrate oxidation rate[16-19] or made more effective for a given mitochondrial respiration rate.[20] Conversely, increased demands for heat production may lead to greater proton leakage and decreased coupling. This has been studied especially in the mitochondria-rich brown adipose tissue (BAT) of mammals where uncoupling is the basis for non-shivering thermogenesis.[10] However, analogous processes are reported for other tissues and organs, both in mammals[21-24] and in organisms that lack BAT.[19,25,26]

Studies of functional changes to cellular respiration are scarce compared to work at the organismal level, especially in wild animals. This is probably because such studies often require terminal sampling, administration of anesthetics to collect biopsies, and swift handling of samples to ensure cells stay alive; all of which may be difficult to reconcile with work on wild species. However, birds and some other nonmammalian vertebrates do not lose the nucleus and organelles present in the hemocytoblast during erythroblast maturation,[27,28] and so contain mitochondria in their red blood cells (RBC). Thus, mitochondrial function can be assessed even from small (eg, 25-50 μl) blood volumes,[29-32] which is not possible when measurements are performed on other mitochondrial-rich blood cells (as is necessary in mammals[33,34]). Hence, sample collection can be minimally invasive and non-terminal, even in small species, facilitating application in field settings and in non-model taxa (eg, 32,35). While much is still unknown about the role and regulation of RBC mitochondria relative to mitochondria in other tissues, studies indicate that RBC respiration varies in line with shorter-term changes to organismal-level energy expenditure[32,35] and over longer time periods in line with an age-related decline in metabolic rate.[29] Thus, there is evidence that measurement of mitochondrial function in RBC can provide a minimally invasive marker of respiratory responses in tissues with more clearly defined metabolic roles. To understand if this applies also to cold adaptation, we need to measure mitochondrial function over environmental gradients known to cause organismal level changes to thermo-physiological parameters.

We studied seasonal variation in RBC respiration in three sympatric small bird species—Coal tits (*Periparus ater*), Blue tits (*Cyanistes caeruleus*), and Great tits (*Parus major*)—that wintered sympatrically in a temperate region of Western Scotland. These birds are within the same taxonomic family, but differ more than twofold in body mass (Coal tits: 8-10g; Blue tits: 9-13g; Great tits: 17-22g; Table 1). Metabolic intensity and, hence, heat loss rate should therefore be highest in Coal tits and least in Great tits. Moreover, Great tits are behaviorally dominant over Blue tits that are dominant over Coal tits (though the latter increases predictability of food supply by hoarding).[36] It is reasonable that these factors might call for differential seasonal adaptation of thermogenic performance at the organismal level, which could be coupled to corresponding changes of cellular bioenergetics to fuel thermogenesis. If any seasonal change in RBC mitochondrial traits were directly or indirectly related to analogous responses in tissues and organs with clearly defined roles in thermoregulation, we predicted that winter-acclimatized birds would show higher mitochondrial volume, increased coupling of electron transport to ATP production (ie, more efficient oxidative phosphorylation), and increased maximum ETS capacity, in line with a higher demand for substrate

TABLE 1 Overview of the study species when measuring seasonal acclimatization of red blood cell mitochondrial respiration at a temperate site in Western Scotland

		
Coal tit (*Periparus ater*)	**Blue tit (*Cyanistes caeruleus*)**	**Great tit (*Parus major*)**
Body mass ± SEM: 9.2 ± 0.1 g Range (8.1 to 10.4 g)	Body mass ± SEM: 11.0 ± 0.1 g (Range: 8.8 to 12.8 g)	Body mass ± SEM: 19.3 ± 0.2 g (Range: 16.8 to 21.7 g)
$N_{autumn} = 22$ $N_{winter} = 16$	$N_{autumn} = 39$ $N_{winter} = 56$	$N_{autumn} = 33$ $N_{winter} = 31$

Note: See Table S1 for further details on sample sizes for each species, season, and respiration trait. The scale reference placed on each bird is 10 × 10 mm.
Abbreviation: SEM, Standard Error of Mean.

delivery to heat producing tissues. If changes to cell respiration reflected organismal energy demands, we also predicted that seasonal changes to trait values would be the largest in Coal tits and the smallest in Great tits.

2 | MATERIALS AND METHODS

2.1 | Study area and bird capture

Fieldwork was performed at the Scottish Centre for Ecology and the Natural Environment (SCENE), near Rowardennan in Western Scotland (56°7'43"N, 4°36'49"W) during autumn to winter 2018-2019. The study area was part of a continuously forested woodland area dominated by oak (*Quercus robur*) in the canopy layer and a sparse understory composed mainly of rowan (*Sorbus aucuparia*), birch (*Betula pendula*), sallow (*Salix* spp.), and holly (*Ilex aquifolium*). Two feeders containing peanut granules (Haith's, Grimsby, UK) were installed 1.5 m above the ground and 200 m apart in early September 2018 to attract resident and wintering birds. The feeders were replenished at least once daily to ensure food was continuously available until the end of the study in March 2019.

We mist-netted sympatric Coal tits, Blue tits, and Great tits at these feeders from 1 h after sunrise to 1 h before sunset during a period in both early autumn (1-14 October 2018) and late winter (19-28 February 2019). An overview of species characteristics and sample sizes for each study period are presented in Table 1. Detailed information on sample sizes per species, season, and respiration trait is available in Table S1 (see Electronic Supplementary Materials, ESM, 1). All birds were ringed with a uniquely numbered aluminum ring issued by the British Trust for Ornithology, and also with unique combinations of either one colored passive integrated transponder (EM4102, Eccel Technology, Leicester, UK) and two plastic color ring, or three color rings (for use in a different study). We then measured tarsus length (±0.1 mm), wing length (±0.5 mm), and body mass (±0.1 g), and collected a blood sample ≤10% of total blood volume (range: 75-150 μL) from the jugular vein in a heparinized Eppendorf tube. Samples were kept on ice until further processing 10-40 minutes later. Birds were held until recovered and were then released close to the capture site.

Weather data for the study periods were available from a MiniMet Automatic Weather Station (Skye Instruments, Powys, UK) in the immediate vicinity (20 m) of the bird feeders. Air temperature ranged from 2.1 to 19.0°C (mean ± SD: 10.7 ± 3.3°C) in early autumn, and 0.5 to 13.1°C (mean ± s.d.: 7.2 ± 2.8°C) in late winter. Photoperiod ranged from 10.5 to 11.6 hours in autumn and 9.9 to 10.6 hours in winter.

2.2 | RBC homogenate preparation and measurement of mitochondrial respiration

The blood samples were first centrifuged at 3000 g for 10 minutes at room temperature (18-20°C) to separate plasma

from the RBCs. The plasma was then removed and the pellet was re-suspended in 500 μL cool (10-15°C) respirometry buffer (MiR05: 0.5 mmol L^{-1} of EGTA, 3 mmol L^{-1} of $MgCl_2$, 60 mmol L^{-1} of K-lactobionate, 20 mmol L^{-1} of taurine, 10 mmol L^{-1} of KH_2PO_4, 20 mmol L^{-1} of HEPES, 110 mmol L^{-1} of sucrose, and free fatty acid bovine serum albumin (1 g L^{-1}), pH 7.1). We then centrifuged the samples at 1000 g for 5 minutes and discarded the supernatant, leaving intact RBCs. These were kept at room temperature until used 1-10 minutes later.

Mitochondrial respiration was measured at a simulated avian body temperature (41°C)[37] following Stier et al[31] with slight modifications similar to Dawson and Salmón.[29] After washing, we re-suspended 40 μL (Coal tits, Blue tits) or 60 μL (Great tits) of the RBC in 750 μL of MiR05 kept at 41°C. The remaining RBCs were stored at −80°C until measurement of citrate synthase (CS) content (below) in May 2019. We used a larger RBC volume in the Great tits since our pilot work showed that 60 μL of samples were required to achieve an optimal signal in this species due to a lower overall respiration rates (see Results). The RBC resuspension was then added to 1.35 mL of MiR05 (final volume of 2 mL) in the respiration chamber of an Oxygraph O2k high-resolution respirometer (Oroboros Instruments, Innsbruck, Austria). The chamber was left open for 1-2 minutes until O_2 concentration was >140 nmol mL^{-1}. O_2 concentration was maintained at, or above, this level throughout the assay.

Mitochondrial respiration rate was measured as the rate of decline in O_2 concentration in the chamber. Once the O_2 consumption signal had stabilized upon closing the chamber, we recorded the baseline respiration of the RBCs with their endogenous substrates ("ROUTINE," ie, the baseline rate of oxygen consumption during the production of ATP) for 2-3 minutes. We then added 2.5 μmol L^{-1} of oligomycin to inhibit mitochondrial ATP synthesis ("LEAK"; ie, the level of mitochondrial respiration required to offset the leak of protons that occurs across the inner mitochondrial membrane when no ATP is being produced). This was followed by 0.25 μmol L^{-1} of the mitochondrial uncoupler carbonyl cyanide-p-trifluoro-methoxyphenyl-hydrazone (FCCP) to stimulate maximum respiration capacity of the electron transport system when uncoupled from F_1F_0-ATP synthase ("ETS"). The concentration of FCCP yielding maximal respiration was determined by titration of 0.1 μL FCCP aliquots in pilot studies. Finally, we added 2.5 μmol L^{-1} of antimycin A (an inhibitor of mitochondrial complex III) to account for non-mitochondrial O_2 consumption. This respiration recorded in the presence of antimycin A was subtracted from all other respiration rates before analyses. Mitochondrial respiration used to drive oxidative phosphorylation during ATP synthesis ("OXPHOS") was inferred from the difference between ROUTINE and LEAK.

2.3 | Citrate synthase content measurement

Citrate synthase (CS) activity is a commonly used marker of mitochondrial volume.[38] Maximal CS activity in RBCs was assayed at 41°C following Dawson et al.[11] Briefly, thawed samples were kept on ice and homogenized in 2.5 volumes of homogenizing buffer [100 mmol L^{-1} KH_2PO_4 buffer, pH 7.2, containing 1 mmol L^{-1} EGTA, 1 mmol L^{-1} EDTA and 1 mmol L^{-1} phenylmethylsulfonylfluoride (PMSF)]. The homogenates were then centrifuged at 1000 g at 4°C and the resultant supernatant was used in the assay. We performed the measurements at 412 nm [ε = 14.15 (mmol $L^{-1})^{-1}$] in 100 mmol L^{-1} KH_2PO_4 (pH 7.2), 0.15 mmol L^{-1} acetyl-coA, 0.15 mmol L^{-1} 5,5′-dithiobis-2-nitrobenzoic acid, and 0.5 mmol L^{-1} oxaloacetate (omitted in blank). The assay was run using a SpectraMaxPlus 384 spectrophotometer (Molecular Devices), and data were analyzed using the accompanying SoftMax Pro 6.3 program. All samples were run in triplicates and the means were used in all analyses (repeatability, $r = 0.97$; $F_{203,\ 408} = 106.1$; $P < .001$).

2.4 | Data analyses

We excluded all data from individuals where respiration after addition of 2.5 μmol L^{-1} oligomycin was higher than endogenous respiration (8 of 205 observations: Coal tit = 1; Blue tit = 5; Great tit = 2). We also excluded ETS data in cases where 0.25 μmol L^{-1} of FCCP inhibited respiration (ie, ETS < ROUTINE; 33 of the remaining 197 observations: Coal tit = 6; Blue tit = 22; Great tit = 5). Cases with negative LEAK respiration, which occurred in 18 Great tits in autumn, were manually assigned a value of 0. We then calculated three flux control ratios (FCR) for: (a) coupling efficiency of ATP production during endogenous respiration (ie, LEAK/ROUTINE; $FCR_{L\div R}$, so that low values indicate a tighter linkage of mitochondrial respiration to ATP production, with little leakage of protons across the inner mitochondrial membrane); (b) coupling efficiency of ATP production during a stimulated cellular state (ie, LEAK/ETS; $FCR_{L\div ETS}$, so that low values again indicate little proton leakage even when the electron transport system is maximally stimulated); and (c) the fraction of maximum working capacity used during endogenous respiration (ie, ROUTINE/ETS; $FCR_{R\div ETS}$). Original and final sample sizes for each respiration metric are presented in Table S1.

All statistical analyses were performed using R 3.6.1.[39] We analyzed variation in all mitochondrial respiration metrics (normalized to mitochondrial volume), CS activity, and flux control ratios, with "season," "species," and "season × species" as fixed variables. The latter was the critical test for differential seasonal adaptation in the study species. Because

TABLE 2 Test statistics, degrees of freedom, corresponding *P* values, and final model estimates, when analyzing the seasonal variation in mitochondrial respiration in sympatric Coal, Blue, and Great tits in Western Scotland

Models and parameters	Estimates (SEM)	*df*	*F*	*P*
CS Activity (μL × min^{-1} × μL RBC^{-1})				
Season (Autumn or Winter)		1, 193	18.88	**<.001**
Season = Autumn [A]	3.50 (0.07)			
Season = Winter [B]	3.81 (0.07)			
Species (Coal, Blue, or Great tit)		2, 193	86.08	**<.001**
Species = Coal tit [A]	2.63 (0.11)			
Species = Blue tit [B]	4.02 (0.07)			
Species = Great tit [C]	4.30 (0.08)			
Species × Season		2, 191	0.75	.4721
ROUTINE ($\dot{V}_{O_2}$× CS $Activity^{-1}$)				
Species (Coal, Blue, or Great tit)		2, 194	61.22	**<.001**
Species = Coal tit [A]	0.31 (0.01)			
Species = Blue tit [B]	0.18 (0.01)			
Species = Great tit [C]	0.11 (0.01)			
Season (Autumn or Winter)		1, 193	1.61	.2056
Species × Season		2, 191	0.57	.5646
OXPHOS ($\dot{V}_{O_2}$× CS $Activity^{-1}$)				
Species (Coal, Blue, or Great tit)		2, 194	23.31	**<.001**
Species = Coal tit [A]	0.17 (0.01)			
Species = Blue tit [B]	0.09 (0.01)			
Species = Great tit [B]	0.09 (0.01)			
Season (Autumn or Winter)		1, 193	3.57	*.0601*
Species × Season		2, 191	0.63	.5321
ETS ($\dot{V}_{O_2}$× CS $Activity^{-1}$)				
Species (Coal, Blue, or Great tit)		2, 160	68.49	**<.001**
Species = Coal tit [A]	0.41 (0.02)			
Species = Blue tit [B]	0.25 (0.01)			
Species = Great tit [C]	0.17 (0.01)			
Season (Autumn or Winter)		1, 160	0.35	.5540
Species × Season		2, 158	1.34	.2647
LEAK ($\dot{V}_{O_2}$× CS $Activity^{-1}$)				
Season (Autumn or Winter)		1, 193	39.36	
Season = Autumn [A]	0.065 (0.004)			
Season = Winter [B]	0.010 (0.004)			
Species (Coal, Blue, or Great tit)		2, 193	113.77	**<.001**
Species = Coal tit [A]	0.139 (0.006)			
Species = Blue tit [B]	0.080 (0.004)			
Species = Great tit [C]	0.028 (0.005)			
Species × Season		2, 191	0.55	.5757
$FCR_{LEAK \div ROUTINE}$				
Season (Autumn or Winter)		1, 191	100.61	**<.001**
Species (Coal, Blue, or Great tit)		2, 191	71.17	**<.001**

(Continues)

TABLE 2 (Continued)

Models and parameters	Estimates (SEM)	*df*	*F*	*P*
Species × Season		2, 191	6.59	**.0017**
Species = Coal tit				
Season = Autumn [A]	0.43 (0.03)			
Season = Winter [B]	0.53 (0.03)			
Species = Blue tit				
Season = Autumn [A]	0.39 (0.02)			
Season = Winter [B]	0.54 (0.02)			
Species = Great tit				
Season = Autumn [A]	0.08 (0.02)			
Season = Winter [B]	0.36 (0.02)			
$FCR_{LEAK \div ETS}$				
Season (Autumn or Winter)		1, 160	71.69	**<.001**
Season = Autumn [A]	0.21 (0.01)			
Season = Winter [B]	0.35 (0.01)			
Species (Coal, Blue, or Great tit)		2, 160	61.10	**<.001**
Species = Coal tit [A]	0.36 (0.02)			
Species = Blue tit [A]	0.33 (0.01)			
Species = Great tit [B]	0.15 (0.01)			
Species × Season		2, 158	2.69	*.0710*
$FCR_{ROUTINE \div ETS}$				
Season (Autumn or Winter)		1, 158	0.05	.8206
Species (Coal, Blue, or Great tit)		2, 158	0.50	.6050
Species × Season		2, 158	3.08	**.0488**
Species = Coal tit				
Season = Autumn [A]	0.68 (0.03)			
Season = Winter [B]	0.80 (0.02)			
Species = Blue tit				
Season = Autumn [A]	0.74 (0.03)			
Season = Winter [A]	0.69 (0.02)			
Species = Great tit				
Season = Autumn [A]	0.69 (0.03)			
Season = Winter [A]	0.70 (0.03)			

Note: Significant (ie, $P < .05$) effects are printed in bold font. Effects for which $0.05 \geq P < .10$ are printed in italic font. Different letters within brackets denote significantly different factor level contrasts as inferred from post hoc tests. Estimates for main effects were not provided when the interaction was significant.

Abbreviations: CS, citrate synthase; FCR, flux control ratio; RBC, Red Blood Cell; SEM, standard error of mean.

some birds (3% of total) were measured in both seasons, we fitted both linear models (lm function in R Base) and linear mixed effects models with a random intercept for "bird ID" (lmer function in the lme4 package).[40] The more complex mixed effects models never improved fit (ie, $AIC_{lmer\text{-}lm} \leq 2$; range: −0.48 to 2.00), so we proceeded with linear models. Non-significant (ie, $P > .05$) terms were removed based on backward elimination, starting with the interactions. Estimates were derived using the emmeans package.[41] When the interaction between season and species was significant, we performed post hoc tests between seasons within species ("pairs" function, emmeans package).

3 | RESULTS

Means (±SEM) and ranges of all respiration metrics and CS activity are presented for each species and season in Table S1. Statistics and final model estimates are presented in Table 2.

3.1 | RBC respiration rate

When adjusting the mitochondrial respiration rates for unit volume of RBC, we found pronounced seasonal increases in all functional traits except OXPHOS (ie, the respiration used to drive ATP production). These responses, which were frequently species-specific, are detailed in ESM 2. When instead adjusting mitochondrial respiration rate for variation in mitochondrial volume (as inferred from CS activity), seasonal changes in all but one functional trait (LEAK; see below) disappeared. However, CS-adjusted ROUTINE (ie, the oxygen consumption of the RBC mitochondria while routinely producing ATP) varied between the species, being the highest in Coal tits, lower in Blue tits, and lower still in Great tits (Table 2; Figure 1A). CS-adjusted OXPHOS followed a broadly similar pattern, being about twice as high in Coal tits compared to Blue and Great tits (that did not differ) (Table 2; Figure 1B). Maximum uncoupled working capacity of the mitochondria (ie, ETS) also did not vary between seasons, but was 2.5 and 1.6 times higher in Coal than in Great and Blue tits, respectively, and 1.5 times higher in Blue tits compared to Great tits (Table 2; Figure 1C). In contrast to the other mitochondrial respiration traits, LEAK (ie, the part of mitochondrial respiration that compensates for the leak of protons across the inner mitochondrial membrane) was higher in winter than in autumn (by a factor 1.5) (Table 2; Figure 1D). When averaging over seasons, CS-adjusted LEAK also differed between the species, being the highest in Coal tits and the lowest in Great tits with Blue tits being intermediate between the two (Table 2; Figure 1D).

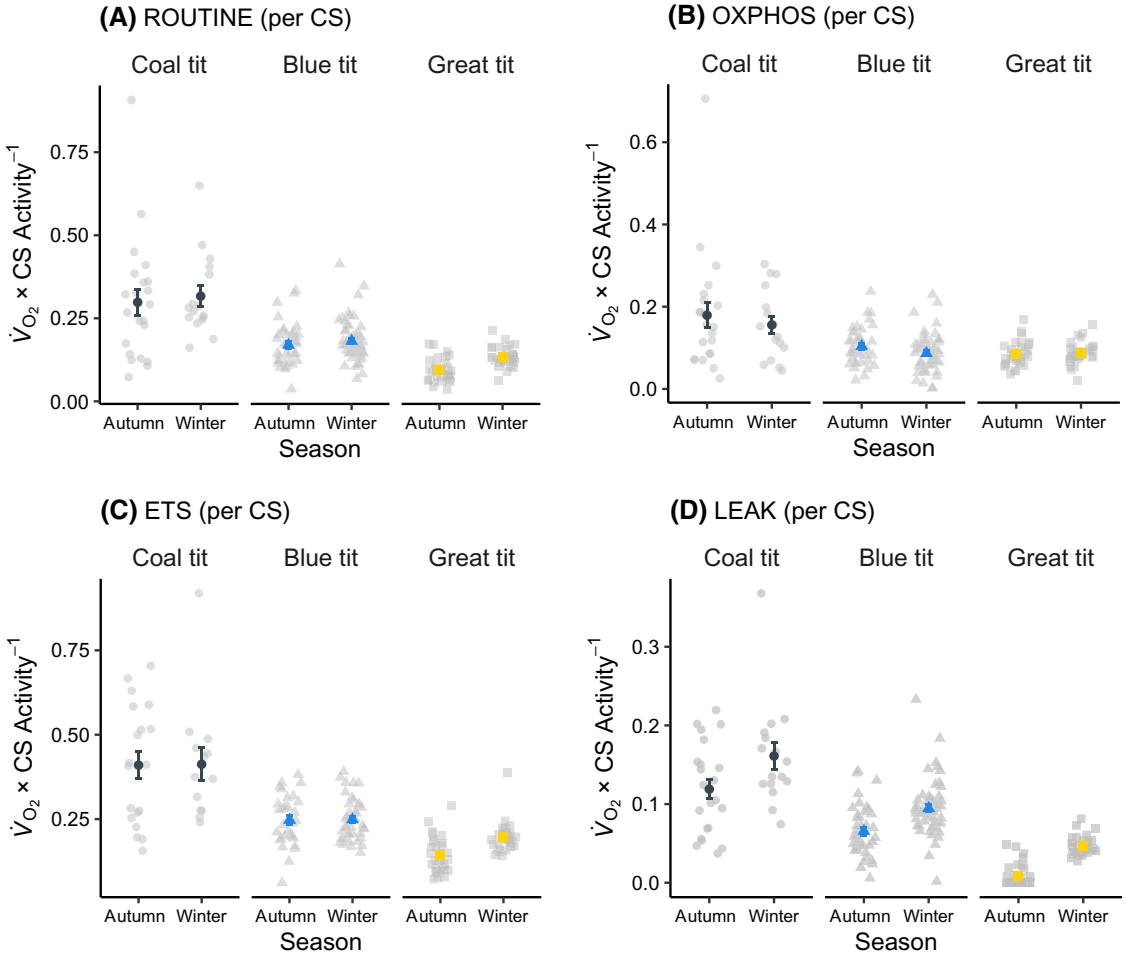


FIGURE 1 Seasonal variation in mitochondrial respiration in intact red blood cells in three sympatric, free-ranging, bird species. The figure shows mean ± SEM. oxygen consumption (pmol $O_2 \times s^{-1} \times \mu L\ RBC^{-1}$) per unit citrate synthase (CS) activity ($\mu mol \times min^{-1} \times \mu L\ RBC^{-1}$), which is an index of mitochondrial volume. The panels show mitochondrial respiration states in Coal, Blue, and Great tits in Western Scotland in early autumn and late winter. A, baseline ("ROUTINE") oxygen consumption during oxidative phosphorylation (ie, while ATP is being produced); (B) oxygen consumption devoted to ATP production alone ("OXPHOS"); (C) maximum working capacity of the electron transport system when uncoupled from ATP production ("ETS"); and (D) the part of ROUTINE that is attributed to offsetting the leak of protons across the inner mitochondrial membrane and so is uncoupled from ATP production ("LEAK"). Gray plotting symbols show raw data. Sample sizes are presented in Table S1, and statistics are presented in Table 2

3.2 | Citrate synthase activity

Citrate synthase activity, a commonly used marker of mitochondrial volume, differed between the species, being some 37% lower in Coal tits than in Blue and Great tits (which had similar CS activity) (Table 2; Figure 2). On average, activity was somewhat upregulated (by a factor 1.1) in winter compared to autumn (Table 2; Figure 2). This seasonal change did not differ between the species (ie, season × species: $P = .5$).

3.3 | Flux control ratios

Seasonal changes in $FCR_{L \div R}$, that is, the coupling efficiency of ATP production during the endogenous cellular state, varied between species. In Coal and Blue tits, LEAK constituted some 50% of endogenous respiration throughout the study, but there was decreased coupling in winter in both species (Table 2; Figure 3A). Great tits had more tightly coupled mitochondria throughout, and also showed a considerably more pronounced seasonal response. Accordingly, LEAK only made up 8% of ROUTINE in Great tits in autumn, but this value increased to 36% in winter (ie, an increase by a factor 5.1) (Table 2; Figure 3A). $FCR_{L \div ETS}$, which is indicative of coupling efficiency during a maximally stimulated state, was about twice as high (2.3 fold) in Coal and Blue tits compared to Great tits, and higher (1.7 fold) in winter than in autumn (Table 2; Figure 3B). $FCR_{R \div ETS}$, which is indicative of how much the cell is respiring in its endogenous state relative to maximum working capacity, showed different seasonal responses in the different species (Table 2; Figure 3C). Thus, $FCR_{R \div ETS}$ did not change between autumn and winter in Blue and Great tits, but was significantly higher (by a factor 1.2) in winter-adapted compared to autumn-adapted Coal tits (Table 2; Figure 3C).

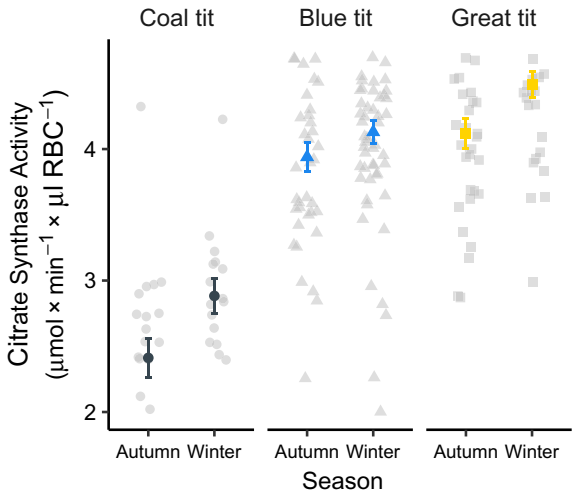


FIGURE 2 Seasonal variation in citrate synthase activity (a proxy for mitochondrial volume). The figure shows mean ± SEM citrate synthase activity in intact red blood cells of sympatric Coal, Blue, and Great tits in the beginning of autumn and in late winter in Western Scotland. Gray plotting symbols show raw data. Sample sizes are presented in Table S1, and statistics are presented in Table 2

4 | DISCUSSION

The most prominent functional change in mitochondrial respiration between autumn and winter was an increase in LEAK respiration that was visible both at the level of the RBC (Figure S1D) and the mitochondrion (Figure 1D), and which led to a seasonal reduction in coupling efficiency (Figure 3A). Thus, bird blood was more thermogenic in winter compared to autumn, at the possible expense of reduced ATP production per O_2 molecule consumed. This is in line with the notion that seasonal acclimatization in small birds acts more strongly on thermogenic performance[7] and not (unlike in larger birds; eg,[4,42]) so much on energy conservation. Had that been the case, we would have expected winter phenotypes to show improved mitochondrial coupling, as is common, for example, in fasting animals.[16,18,43,44] A thermoregulatory role for LEAK respiration in RBCs could also explain why proton conductance decreased with increasing body size at the interspecific level (Figure 1D, Figure S1D) in line with differences in metabolic demands. If increased LEAK respiration in RBCs is mirrored in the phenotypic responses of other tissues such as skeletal muscles, then winter-adapted birds might be able to partly augment thermostatic costs without increasing shivering, thereby escaping some of the possibly negative fitness consequences of increased ROS production[45] that are inherent to oxidative phosphorylation.[46]

While we cannot exclude the possibility that seasonal changes in LEAK reflected some other aspect of seasonal biology, it is tempting to speculate that this was indeed causally related to seasonally declining temperatures (either experienced or anticipated). Cold-induced increases in proton conductance have been found in skeletal muscle, liver, and brain tissue in other studies on endotherms[25,47,48] and are often interpreted as a mechanism for augmenting local thermogenesis.[22-24] On the other hand, the seasonal switch in tits from an insect-dominated summer- to a seed-dominated winter diet is associated with a change in the composition of circulating fatty acids,[49] which could affect leakiness of the mitochondrial membrane. However, of the circulating fatty acids that show seasonally varying abundance and also have a demonstrated mitochondrial uncoupling action in other species (ie, eicosapentaenoic acid [EPA], docosahexaenoic acid [DHA], arachidonic acid [AA], and palmitate),[50,51] only one (AA) was more abundant in blood plasma in winter (the others being more abundant in summer).[49] Thus, it is difficult to speculate on causality at this stage. This should instead be explored in future work.

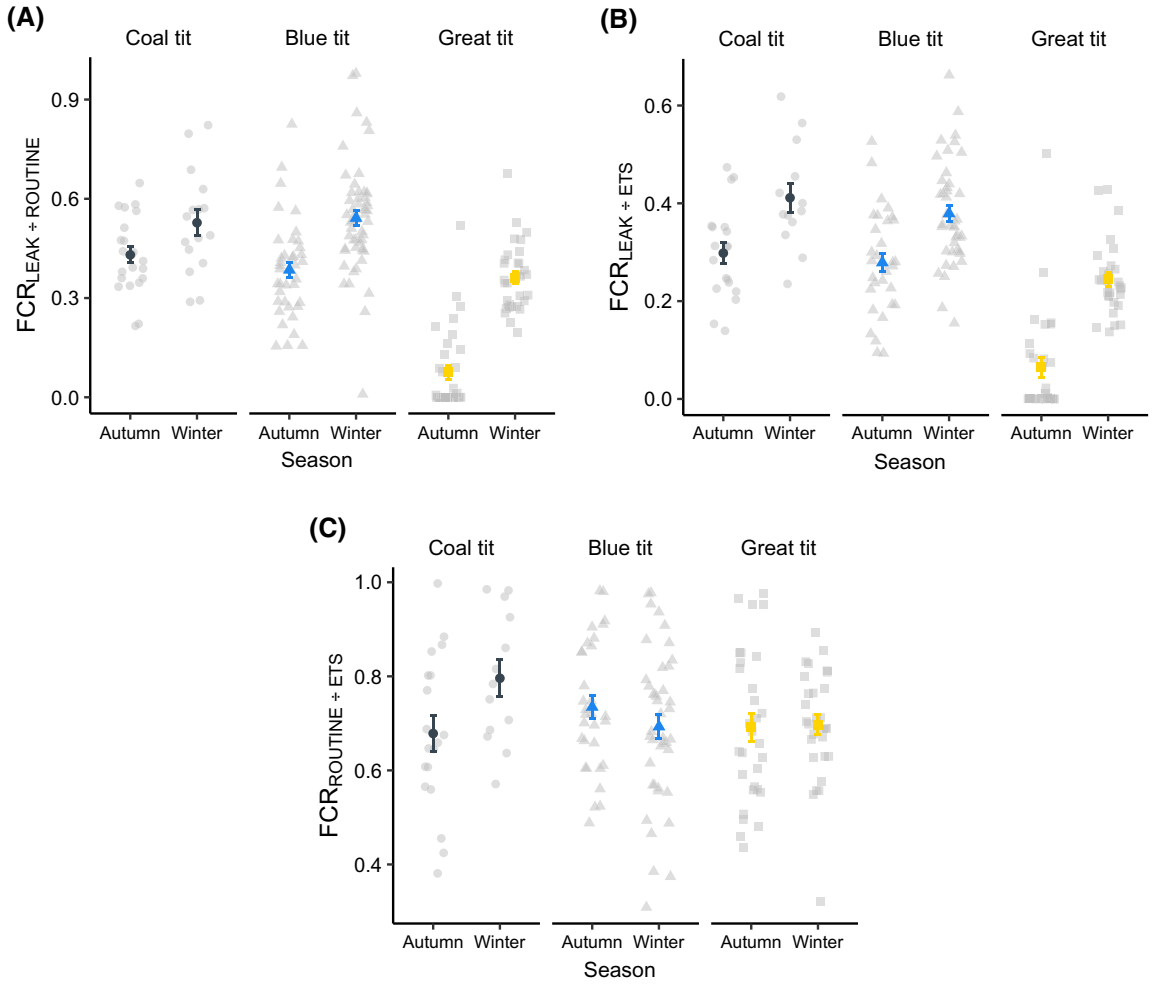


FIGURE 3 Flux control ratios for mitochondrial respiration in autumn and winter in three sympatric, free-ranging, bird species in Western Scotland. The figure shows mean ± SEM flux control ratios (FCR) in intact red blood cells for: A, The fraction of endogenous (ROUTINE) respiration that is linked to LEAK respiration; an index of coupling efficiency of electron transport and ATP production. B, The proportion of maximum mitochondrial working capacity (ETS) that is attributable to LEAK respiration; an index of coupling efficiency during a stimulated cellular state. C, How intensively the mitochondria are respiring under endogenous cellular conditions (ROUTINE) relative to their maximum working capacity (ie, ETS). Gray plotting symbols show raw data. Sample sizes are presented in Table S1, and statistics are presented in Table 2

There was a pronounced upregulation of ROUTINE respiration per RBC in all three species between autumn and winter (1.1- to 1.6-fold; Table S1). This is a commonly observed seasonal response in metabolic or thermogenic tissues and organs, such as in the liver, heart, and in skeletal muscles (eg, 13,14,52). In two of the three species, mitochondrial respiration per RBC also had higher uncoupled respiratory capacity (ie, ETS was higher; Figure S1C) in winter compared to autumn. These changes are in line with the temperature-driven increases in whole-animal metabolic rate and thermogenic performance that is part of winter acclimatization in birds.[53-55]

The seasonal change in CS activity indicated that the birds increased RBC mitochondrial volume between autumn and winter (cf. 38) (Figure 2). When we adjusted he respiration rates for mitochondrial content, all seasonal changes but that in LEAK (discussed above) disappeared (Figure 1A-D). Thus, the seasonal increase in endogenous respiration (ie, ROUTINE) and maximum working capacity (ie, ETS) in the RBCs was mostly a reflection of higher mitochondrial volume in winter and not a result of a seasonal increase in mitochondrial respiratory capacity. Mitochondrial volume in the RBCs also varied between species, with Coal tits having only about 60% of the CS activity of that in Blue and Great tits (Figure 2). Lower mitochondrial volume could explain why Coal tits showed higher CS -corrected substrate oxidation rate compared to the other two species (Figure 1B), such that there was no interspecific difference in OXPHOS per RBC (Figure S1B; Table 2), and why Coal tit mitochondria seemed to work at a higher proportion of maximum in winter than mitochondria from either Blue or Great tits (Figure 3C).

In contrast to our predictions, the extent of seasonal acclimatization of RBC respiration was not a simple body mass relationship. Instead, the phenotype of the smallest study species

(Coal tit) was characterized by low mitochondrial content but higher respiratory capacity. In contrast, the largest species studied (Great tit) had high mitochondrial content and mitochondria with a low respiration rate. The intermediately sized Blue tit, which is still markedly smaller than the Great tit (Table 1), also had a high content/low respiration phenotype. Across all species studied here, the blood was more thermogenic in winter compared to summer, both at tissue (RBC) and organelle (mitochondrion) levels. If this indeed reflected an adaptive response, it is possible that functional changes to mitochondrial respiration might help the bird meet thermostatic costs to low winter temperatures. It is encouraging that the seasonal responses of RBC respiration are broadly similar to those in organs and tissues with clearly defined roles in thermoregulation. This adds to the body of evidence suggesting that RBC mitochondria may be suitable markers for minimally invasive insights into many aspects of eco-evolutionary research on nonmammalian vertebrates, such as in aging and reproductive trade-offs.[22,32,35] To further our knowledge of how RBC respiration is regulated and how it has evolved, there is now a need for interspecific comparisons across seasons and a range of species-specific phenotypes over broad geographic ranges, as well as for studies gauging the relationship between RBC respiration and respiration in other tissues (but see 31) both as simple bivariate relationships and as correlated responses to appropriate manipulation of the environment.

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ETHICS

The experimental procedures were covered by project licenses PPL70/7899 and P6859F36E issued by the Home Office, UK. AN was licensed to perform all aspects of the practical work (Home Office, personal license no. I47473045). Capture and ringing of birds were permitted by the British Trust for Ornithology (BTO) (license no. C6620, to AN). Color ringing and deployment of PIT tags were approved by BTO (under a permit issued to Stewart White, University of Glasgow, ringing license no. A5616).

CONFLICT OF INTEREST

The authors declare that they have no competing or financial interests.

AUTHOR CONTRIBUTIONS

AN, NBM, and DJM conceived the study and designed the protocol together with NJD. AN performed the fieldwork with the help from JLP, AH, and DJM. AN and NJD performed the laboratory work. AN analyzed the data, produced the graphic material, and wrote the first draft. This was reviewed and edited by all authors. AN, NBM, and DJM procured the funding. All authors approved the final version of the manuscript and agreed to be accountable for all contents.

DATA AVAILABILITY STATEMENT

Data are appended in the Electronic Supplements (ESM 3).

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SUPPORTING INFORMATION

Additional Supporting Information may be found online in the Supporting Information section.

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