Plasticity of mitochondrial function restores fuel production during rest-phase hypothermia in a winter bird

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AUTHOR CONTRIBUTIONS: AN conceived the idea and designed the protocol with input from EE. AN performed the fieldwork, and CCGD, IM and AN performed the laboratory work. All authors interpreted the results. CCGD and AN analysed the data and wrote the first draft. CCGD produced the graphic material. All authors critically revised and edited the manuscript. AN procured funding.

ABSTRACT

Many small animals routinely regulate resting body temperature several degrees below active-phase levels (rest-phase hypothermia), which reduces heat transfer rate and tissue oxygen demand and so may confer energy savings. Small birds that winter at high latitude show limited capacity for rest-phase hypothermia, meaning they cannot avoid upregulating heat production when facing winter cold. Substrates for upholding body temperature are provided by mitochondria in the form of adenosine triphosphate (ATP), but mitochondrial respiration is probably lower during hypothermia because of the temperature-dependence of biological processes (i.e., Q_{10} effect). Thus, there could be a conflict between increased organismal fuel demand and a lower mitochondrial capacity to provide it during rest-phase hypothermia. We studied this enigma by assessing mitochondrial function in the blood cells of wintering great tits (*Parus major*) that had spent the preceding night in warm, mild, or cold temperatures, at each of a hypothermic and a normothermic thermal state *in vitro*. Hypothermia reduced mitochondrial respiration by 13 %. However, this did not affect respiration allocated to oxidative phosphorylation, because hypothermia was also associated with a reduction in non-phosphorylating respiration, from 17 % in normothermia to 4 % in hypothermia. The proportion of non-phosphorylating respiration also decreased in response to colder night temperatures Thus, the coupling efficiency between electron transport and ATP production in maximally stimulated cells increased from 91% in normothermia to 98% in hypothermia. Our study shows that mitochondrial function can be highly plastic on short temporal scales. Thus, functional changes in the electron transport system might safeguard ATP production at lower tissue temperatures and when organismal demand for fuel increases in cold winter temperatures.

Keywords: blood cells, body temperature, cell respiration, great tit, mitochondria, thermoregulation, winter

INTRODUCTION

Mammals and birds rely mostly on endogenous heat production to thermoregulate. An endothermic lifestyle carries many benefits, such as the possibility of sustaining high work rate (largely) independently of variation in air temperature. However, it can be cumbersome to amass the resources needed to support the high metabolic throughput machinery that is required for this to happen. This is the case during high latitude winters, when cold temperature increases the energy cost of staying warm at the same time as hours of daylight for foraging, and food availability itself, reach their annual lows. Animals that remain resident in such biomes throughout the year adapt accordingly by a combination of behavioural plasticity and seasonal morphological and physiological adjustments, rendering winter phenotypes better equipped to produce heat and withstand heat loss see [1,2 for reviews].

Many small to medium-sized endotherms save energy by rest-phase hypothermia, a regulated reduction in resting body temperature body temperature below the euthermic level during the resting phase [3]. This typically occurs in response to various energetic challenges, such as food scarcity or inclement weather, and may reduce metabolic demands by more than 50% [4]. It follows that most animals using rest-phase hypothermia have high metabolic intensity, resultant high heat loss rate, and utilises high-energy or ephemeral food sources [3]. This fits the description of "the little bird in winter" sensu [5], i.e., small (< 20 g) songbirds that are year-round residents at high latitudes where they routinely meet environmental temperatures 50-60 °C below body temperature. Yet, most winter birds in northern forests, such as tits and chickadees, rarely show daily variation in body temperature > 5 °C e.g. [6,7,8] and generally do not exceed 7-8 °C even in severe cold [9-11,]but see [12]. Theoretical models predict even such moderate body temperature reduction can carry survival benefits [13,14]. However, the modest extent of hypothermia means that the little bird in winter can never forego a significant

increase in energetically costly heat production to counter winter cold, even when maximally hypothermic e.g. [15].

Most of the energy used by the working body is in the form of adenosine triphosphate (ATP) that is produced at the expense of oxygen in the mitochondria. This occurs by dissipation of the energy contained in a proton gradient across the inner mitochondrial membrane that is accumulated by the mitochondria's electron transport system (ETS) [16]. Since more energy is needed to stay warm in winter, it is not surprising that many endotherms upregulate mitochondrial content and/or respiration rate in key metabolic tissues when cold exposed [17-20,]e.g. [21]. However, a potential problem for a hypothermic animal is that the activity of the respiratory chain could be slower in a colder body e.g. [22], resulting in a conflict between increased organismal demand for fuel but reduced mitochondrial capacity to provide it. However, not all of the proton motive force generated by the ETS is used to produce substrate, because under the influence of uncoupling proteins and due to spontaneous "slip", protons also leak back into the mitochondrial matrix without generating ATP in the process [23]. This process, known as mitochondrial proton leak, constitutes some 20-25% of resting metabolic rate in endotherms [24,25]. Thus, there could be scope to adjust the coupling of ETS to ATP production according to the animal's need. For example, increased mitochondrial coupling during energy limitation makes ATP production more effective for a given respiration rate [26-29]. It could, thus, be speculated that improved coupling could secure adequate ATP production even when the overall energy expenditure of the cell is reduced, such as when heat production must remain high, but substrate delivery is reduced because rest-phase hypothermia causes the mitochondria to work at a slower pace. There is support for this compensation hypothesis from studies on ectotherms [30-32] but see [33,34]. Similar studies on endotherms suggests that while phosphorylating respiration is often actively suppressed in

hypometabolic animals, non-phosphorylating (i.e., leak) respiration is sometimes suppressed, sometimes unaltered, and sometimes increased reviewed by [35].

Previous endotherm studies have largely been performed in hibernators or in animals capable of deep torpor; thermal states characterised by substantial metabolic depression [4]. In such models, it is likely that the combination of direct thermal suppression and active downregulation of phosphorylating respiration allows for sufficient energy conservation without a concomitant need to tighten coupling. This is not easily extrapolated to the problems faced by the little bird in winter, which must increase energy expenditure sharply to counter cold temperature even when hypothermic. Hence, we studied mitochondrial responses to *in vitro* simulation of rest-phase hypothermia in the great tit (*Parus major* L.) – a small (16-20 g) songbird that winters at high latitude and show pronounced diel variation in body temperature [6,36]. The birds spent the night preceding these measurements in conditions representative of mild or very cold winter temperature, or in thermoneutrality. This allowed us to investigate how short-term changes to the thermal environment affected the mitochondrial response to reduced body temperature. We exploited the fact that birds contain functional mitochondria in their red blood cells, which makes it possible to gain non-terminal insights into mitochondrial function. Previous studies have found that bird blood cell mitochondria respond in the short-term to variation in energetic demands [37-39], over longer time periods in line with age-related metabolic senescence [40] and according to environmental context [20]. If better coupled mitochondria serve as an adaptation to secure a high level of cellular energy production even when the overall energy expenditure of the cell is reduced, we expected that coupling should increase in response to low night-time air temperature and during hypothermia *in vitro*. If, on the other hand, mitochondrial uncoupling can reduce the need for shivering thermogenesis (the requires ATP) or reduce the amount of ROS produced during in

shivering [41,42], hypothermia and low air temperature could also be associated with increased proton leak.

MATERIALS AND METHODS

The experimental design and timeline are outlined in Fig. 1.

Study site and animals

Great tits were caught at two sites in the vicinity of Lund in southernmost Sweden – Räften (n = 37) and Vomb (n = 21) – between January and February 2021. Räften (55° 43'N, 13° 17’E") is an oak (*Quercus robur*) dominated 32 ha woodlot surrounded by arable fields. The Vomb site (55° 39'N, 13° 33'E) is part of a pine (*Pinus sylvestris*) plantation with a rich deciduous understory composed mainly of sallow (*Salix* spp.), birch (*Betula pendula*) and rowan (*Sorbus aucuparia*). Birds in Räften were caught in mist nets close to feeders baited with sunflower seeds that were erected 1.5 m above ground in January 2021. The birds in Vomb were caught shortly before sunrise when roosting in nest boxes. All birds were brought to Lund University within 2 h of capture, where they were housed in cages measuring 77 × 44 × 77 cm (length × width × height) at 5 °C under simulated natural photoperiod and were provided sunflower seeds, peanuts, and water *ad libitum.* One-two days after capture the birds were implanted with a temperature-sensitive PIT tag (BioTherm13, Biomark, Boise, ID, USA) into the intraperitoneal cavity under local anaesthesia (5% lidocaine) as part of a different study. Eight to 34 h later, the birds were put in a 1 L hermetically sealed glass container ventilated with dry air at 400-500 ml / min for measurement of metabolic rate and body temperature in a climate chamber (Weiss Umwelttechnik C180 [Reiskirchen, Germany]) at thermoneutrality (25 °C; n = 20; Andreasson et al., 2020), during a simulated mild winter night (5 °C: n = 19)

and during a simulated very cold winter night (-15 °C; n = 19) for 13-15 h, again for use in the other study.

Ambient temperature in Lund (10-40 km from the study sites) ranged -14 °C to 7 °C during the study period and photoperiod ranged 8:20 h to 10:20 h.

Blood sampling and mitochondrial measurement in whole blood

In the morning after the metabolic measurements, a blood sample ≤ 10% of estimated total blood volume was obtained from the jugular vein using a heparinized syringe within 5 min of removing the bird from the metabolic chamber. The sample was stored at 10-12 °C in 2 mL EDTA (ethylenediaminetetraacetic acid) tubes (BD Vacutainer®, Franklin Lakes, NJ, USA) without agitation until analysed 0.5-2.5 h later.

We measured mitochondrial respiration in whole blood using an Oxygraph O2k high resolution respirometer (Oroboros Instruments, Innsbruck, Austria), following the protocol described in Nord et al.[43]. To test how rest-phase hypothermia affected mitochondrial function, we ran duplicate samples from the same individual simultaneously in each of two thermal states: a representative normothermic daytime bird body temperature (41 °C; henceforth "normothermia") and a representative hypothermic night-time body temperature (35 °C; henceforth "hypothermia") [44] using two respirometers. These were calibrated at assay temperature (i.e., 35 °C or 41 °C) daily, and we changed instrument/assay temperature combinations between days.

The blood samples were allowed 5-10 min on a rotating mixer in room temperature before the start of the assay. Then, we added 50 µL sample to 1.95 mL MiR05 respiration medium (0.5

mM EGTA, 3 mM $MgCl_2$, 60 mM K-lactobionate, 20 mM taurine, 10 mM KH_2PO_4, 20 mM Hepes, 110 mM sucrose, free fatty acid bovine serum albumin (1 g L^{-1}), pH 7.1) that was contained within the respirometer chambers and so was pre-equilibrated at assay temperature. The final volume contained in the respirometer chamber was, thus, 2 mL.

Mitochondrial respiration rate was measured as the rate of decline in O_2 concentration in the chamber. Once the O_2 consumption had stabilized upon closing the chamber, recorded baseline O_2 consumption on endogenous substrates ('ROUTINE') for 2-3 min. Then, we assessed mitochondrial function by sequential addition of three mitochondrial agonists and antagonists. Firstly, we added the ATP-synthase inhibitor oligomycin at a final concentration of 5 µmol L^{-1}, which blocks the process of oxidative phosphorylation ('OXPHOS'). Thus, the remaining O_2 after oligomycin addition is attributed to proton leak ('LEAK'). Next, we added the mitochondrial uncoupler FCCP (carbonyl cyanide-p-trifluoro-methoxyphenyl-hydrazone), which abolishes the proton gradient across the inner mitochondrial membrane, causing the electron transport system to work at maximum capacity to restore the gradient ('ETS'). Because an excessive concentration of FCCP inhibits mitochondrial respiration and can lead to acidification of the cell cytoplasm [16], we first added 1.5 µL of 1 mmol L^{-1} FCCP and then titrated FCCP in 0.5 µL increments until maximum O_2 consumption was reached. The final concentration ranged 1-1.75 µmol L^{-1}. Finally, we added 5 µmol L^{-1} of antimycin A, which blocks Complex III in the electron transport chain. Therefore, the remaining O_2 consumption after antimycin A addition is due to non-mitochondrial respiration.

Statistical analyses

We excluded five of the 57 duplicate samples measured at 41 °C since they were not inhibited by the addition of oligomycin. Thus, the final data set consisted of 57 observations at 35 °C

and 52 observations at 41 °C. We then calculated mitochondrial respiration states, flux control ratios (FCR) and coupling efficiency according to Table 1.

All statistical analyses were performed using R version 4.0.4 (R Core Team, 2020). We used linear mixed models (lmer in the lme4 package) [45] to test how each respiration state and flow control ratio was affected by assay temperature, night temperature and assay temperature × night temperature. Bird ID nested in night temperature was used as a random factor to account for repeated measurements. Final models were derived using backward elimination of non-significant terms based on likelihood ratio tests [46]. Predicted values and their standard errors (s.e.m.) were calculated using the emmeans functions in the emmeans package [47]. Post hoc tests were performed using the pairs function in emmeans.

RESULTS

A representative normothermic experiment is presented in Fig. 2. All details on the statistical tests are presented in Table 2.

The effect of thermal state on ROUTINE was similar in all night temperatures (i.e., assay temperature × night temperature: P = 0.568) and ROUTINE was not affected by night temperature (P = 0.406). However, ROUTINE was 13 % higher in normothermia (0.572±0.020 pmol O_2 s^{-1} μl^{-1}) than at hypothermia (0.551±0.020 pmol O_2 s^{-1} μl^{-1}) (P < 0.001) (Fig. 3A), i.e., endogenous respiration was slower when the blood was colder. OXPHOS was not influenced by the interaction between thermal state and night temperature (P = 0.895) and was also not affected by thermal state (P = 0.450) or night temperature (P = 0.218) alone (Fig. 3B). This was also true for ETS (thermal state × night temperature: P = 0.214; thermal state:

P = 0.480; night temperature: P = 0.308) (Fig. 3C). However, LEAK was markedly affected by assay temperature (P < 0.0001), being more than three times higher during normothermic (0.096±0.004 pmol O_2 s^{-1} μl^{-1}) compared to hypothermic (0.023±0.004 pmol O_2 s^{-1} μl^{-1}) assays (Fig. 3D).

The mitochondria respired at a higher proportion of their maximum capacity when normothermic (0.570±0.013, of 1) than when hypothermic (0.496±0.012) (i.e., $FCR_{ROUTINE/ETS}$ was lower during hypothermia). In other words, hypothermic cells had more surplus respiratory capacity compared to normothermic cells (Fig. 4A). The transition from a normothermic to a hypothermic state also affected the contribution of LEAK to endogenous respiration (i.e., there was a significant change in $FCR_{LEAK/ROUTINE}$; Fig. 4B). Specifically, during normothermia LEAK accounted for nearly 17% of endogenous respiration (0.166±0.007), but in hypothermia LEAK contributed only 4% to ROUTINE (0.042±0.007) (Fig. 4). Coupling efficiency when the cells were induced to work harder (i.e., when maximal respiration was stimulated using FCCP) increased from 91% (0.906±0.004) in normothermia to 98% (0.979±0.004) in hypothermia (Fig. 4C). Thus, the electron transport chain was tighter when in a hypothermic state. When thermal states were accounted for, there was also a tendency for a change to $FCR_{LEAK/ROUTINE}$ in response to night temperature (P = 0.059), whereby LEAK contributed less to ROUTINE after a night in -15°C than after a night at -5 and 25°C, though differences in $FCR_{LEAK/ROUTINE}$ were small between the coldest and warmest night temperatures (Table 2). Fig. 4

DISCUSSION

We tested whether mitochondrial respiration in great tit blood cells varied with thermal state (i.e., normothermia or hypothermia) and air temperature. Endogenous respiration ('ROUTINE') was lower during *in vitro* hypothermia (Fig. 3A). However, the part of respiration devoted to oxidative phosphorylation (OXPHOS; in which ATP is produced) remained constant during the transition from normo- to hypothermia (Fig. 3B). At the same time, non-phosphorylating (i.e., LEAK) respiration decreased threefold (Fig. 3D). As a result, LEAK constituted significantly less of endogenous respiration rate in the hypothermic state (i.e., $FCR_{LEAK/ROUTINE}$ was lower; Fig. 4B) and coupling efficiency when the cells were stimulated to work hard also increased during hypothermia Fig. 4C). Non-uniformity of changes to respiration traits indicates that the mitochondrial response to cooling were actively regulated and not merely a consequence of thermal suppression of the ETS cf. [48,49]. Hence, our study indicates that flexibility in mitochondrial function may allow the little bird in winter to secure sufficient substrate delivery to meet thermoregulatory demands despite a general depression of cellular respiration during hypothermia. This notion should be explored by direct measurement of ATP production and efficiency of phosphorylating respiration in normo- and hypothermic birds. Future studies should also address if the response recorded in blood cells mirrors that in other tissues with more clearly defined roles in energy metabolism and thermogenesis. For example, hibernating or torpid mammals show downregulation of phosphorylating and non-phosphorylating respiration in some tissues (e.g. the liver) [50,51] but not in others (e.g. skeletal muscles) see [35 for a review,51]. In some cases, metabolically suppressed states may even be associated with reduced coupling of the ETS, possibly to aid local thermogenesis in the central nervous system [52].

Reduced LEAK and improved coupling efficiency during hypothermia is in keeping with the hypothesis of biochemical compensation for thermal suppression of cell respiration at lower

body temperature. However, tighter coupling can potentially result in increased production of reactive oxygen species (ROS) as a by-product of electron transport [23,53], provided that the resultant increase in protonmotive force is not dissipated through ATP synthase. Unless quenched, ROS cause oxidative stress with resultant macromolecular damage e.g. [54], which is related to a large number of medical disorders [55]. This has given rise to the "uncoupling to survive" hypothesis, in which mitochondrial ROS are implicated in the aging process [56,57]. It is interesting to speculate that a hypothermic bird might have to accept an oxidative cost to uphold ATP production. This could explain why the suppressing effect of low night temperature on LEAK was only evident at the normothermic assay temperature (Fig. 3D) but stabilised at a constant low level in the hypothermic state. In this context, our study could provide proximate insights into why oxidative stress has been found to be higher in birds that are more hypothermic at night [58]. However, the role of mitochondria as sources of cellular ROS has been contended [59-61] and empirical evidence suggests that the relationship between body temperature, ATP production and ROS generation is not straightforward [29,62,63]. In line with this, even though (pharmacological) uncoupling protects birds from cold-induced oxidative stress [41], the evidence for clear effects thereof on fitness in this taxon are equivocal [64,65]. It is advisable that future studies measure both ATP- and ROS production, and membrane potential, to determine if a hypothermic bird pays an oxidative cost.

We found evidence of improved coupling of the ETS after a 12-14 h acute exposure to lower night-time temperature (Fig. 4A). This contrasts previous studies on birds and mammals, where cold exposure or cold acclimatisation was associated with increased non-phosphorylating respiration or increased expression of uncoupling proteins [66-70]. These responses have been studied most frequently in skeletal muscle, and have been implicated as a transition towards increased reliance on non-shivering thermogenesis, though its occurrence

and significance in the skeletal muscles of birds remains dubious [71] but see e.g. [69,72]. Similar increases in LEAK respiration were also found in the blood cells of three temperate species of tits during the transition from mild autumn to cold winter temperature [20]. It is interesting to note that the studies cited above have measured changes to mitochondrial respiration after longer-term exposure to cold temperature whereas we measured the response to an immediate temperature change. It is possible that the short-term response to colder environmental temperature is a tightening of the ETS to secure substrate delivery, whereas the longer-term response may reflect a balance between the competing demands of producing ATP and keeping ROS levels minimal that could be facilitated by a simultaneous increase of mitochondrial content (e.g., Nord et al. 2021).

This study adds new perspectives to our understanding of how cellular- and whole animal metabolism interact when the little bird in winter adapts to low temperatures. We conclude that, in the short term, blood cell mitochondria maintain phosphorylating respiration by reducing proton leak and increasing coupling efficiency when body or air temperature decreases. It is possible, therefore, that rest-phase hypothermia may come at the cost of increased oxidative stress cf. [58]. It is interesting to note that this acute response to reduced air temperature is opposite to what has been recorded as part of seasonal acclimatisation in the same model system [20] and in response to cold exposure in tissues with more clearly defined metabolic or thermogenic roles in other models (see references above). These striking difference in short- and long-term responses to colder temperature demands a need to study the temporal resolution of blood cell responses in some detail. Even so, this work adds to the body of evidence highlighting the utility of studying blood cells to gain minimally invasive insight when integrating mitochondrial function into the study of functional ecology, evolutionary physiology, and gerontology in birds [37,38,40,73,74]. Future studies should address

how changes to phosphorylating and non-phosphorylating respiration during hypothermia in this model correlates with simultaneous changes to ATP production and ROS generation and how responses in blood cells correlate with those in other tissues. More broadly, it would be relevant to increase taxonomic and phenotypic coverage of these studies, spanning cold-hardy, non-hypothermic, birds e.g. [75] to those that routinely use shallow e.g. [76] or deep torpor [77-79].

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ETHICS

All procedures were approved by the Malmö/Lund Animal Ethics Committee, acting under jurisdiction of the Swedish Board of Agriculture (permit no. 9246-19). Bird ringing was permitted by the Swedish Bird Ringing Centre at Natural History Museum of Sweden (license number 723, to AN).

DATA AVAILABILITY

Data will be deposited in Figshare upon acceptance of the manuscript.

CONFLICT OF INTEREST

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

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TABLES

Table 1. Calculation method and definition of mitochondrial respiration states. Mitochondrial parameters were measured in terms of changes in O_2 concentration after the addition of agonists and antagonists of mitochondrial respiration.

Parameter	Calculation method	Definition
ROUTINE	$R_{Baseline} – R_{AntimycinA}$	Baseline O_2 consumption during oxidative phosphorylation under endogenous cellular conditions.
OXPHOS	$R_{Baseline} – R_{Oligomycin}$	O_2 consumption used to drive oxidative phosphorylation where ATP is produced.
ETS	$R_{FCCP} – R_{AntimycinA}$	O_2 consumption when the electron transport system is operating at its maximum capacity.
LEAK	$R_{Oligomycin} – R_{AntimycinA}$	O_2 consumption needed to compensate for proton leakiness of the mitochondria.
$FCR_{ROUTINE/ETS}$	ROUTINE / ETS	Proportion of ETS maximum working capacity used under endogenous cellular conditions.
$FCR_{LEAK/ROUTINE}$	LEAK / ROUTINE	The proportion of endogenous respiration used to compensate for proton leak under non-stimulated cellular conditions.
Coupling Efficiency	1 – (LEAK / ETS)	Coupling efficiency of ATP production in a stimulated cellular state.

Table 2. Results from final models comparing mitochondrial respiration parameters in great tit whole blood in each of two assay temperatures. Means are presented ± s.e.m. Values for significant effects (i.e., $P \leq 0.05$) are presented in bold font, and values for effects where $0.05 < P < 0.10$ are given in italics. Note that test statistics and parameter estimates for main effects are not provided when interactions are retained in the final model. *$T_{night[-15°C]}$ vs. $T_{night[5°C]}$: $P = 0.058$.

Model and Variable	Estimate (± s.e.m.)	$n_{35°C}$ / $n_{41°C}$	*LRT*	*P*
ROUTINE (pmol O_2 s^{-1} μl^{-1})		57 / 52		
Night Temp × Assay Temp			1.13	0.568
Night Temp (-15°C, 5°C or 25°C)			1.80	0.406
Thermal State			17.77	**< 0.001**
Hypothermia (35°C)	0.505±0.020			
Normothermia (41°C)	0.572±0.020			
OXPHOS (pmol O_2 s^{-1} μl^{-1})				
Night Temp × Assay Temp		57 / 52	0.22	0.895
Thermal State			0.57	0.450
Night Temp (-15°C, 5°C or 25°C)			3.05	0.218
ETS (pmol O_2 s^{-1} μl^{-1})				
Night Temp × Assay Temp		57 / 52	3.08	0.214
Thermal State			0.50	0.480
Night Temp (-15°C, 5°C or 25°C)			2.35	0.308
LEAK (pmol O_2 s^{-1} μl^{-1})				
Night Temp × Assay Temp			3.56	0.168
Night Temp (-15°C, 5°C or 25°C)		57/52	2.08	0.354
Thermal State			77.57	**< 0.001**
Hypothermia (35°C)	0.023±0.006			
Normothermia (41°C)	0.096±0.006			
FCR ROUTINE / ETS				
Night Temp × Assay Temp			0.43	0.807
Night Temp (-15°C, 5°C or 25°C)			3.56	0.169
Thermal State		57/52	37.72	**< 0.001**
Hypothermia (35°C)	0.496±0.012			
Normothermia (41°C)	0.570±0.012			
FCR LEAK / ROUTINE				
Night Temp × Assay Temp			1.81	0.404
Night Temp (-15°C, 5°C or 25°C)			5.65	*0.059*
Night Temp = -15°C [A]*	0.088±0.010	18 / 17		
Night Temp = 5°C [A]	0.122±0.010	19 / 17		
Night Temp = 25°C [A]	0.101±0.010	20 / 18		
Thermal State			95.51	**< 0.001**
Hypothermia (35°C)	0.042±0.007			
Normothermia (41°C)	0.166±0.007			
Coupling Efficiency		57 / 52		
Night Temp × Assay Temp			0.90	0.636
Night Temp (-15°C, 5°C or 25°C)			5.15	*0.076*
Thermal State			92.57	**< 0.001**
Hypothermia (35°C)	0.979±0.004			
Normothermia (41°C)	0.906±0.004			

FIGURES

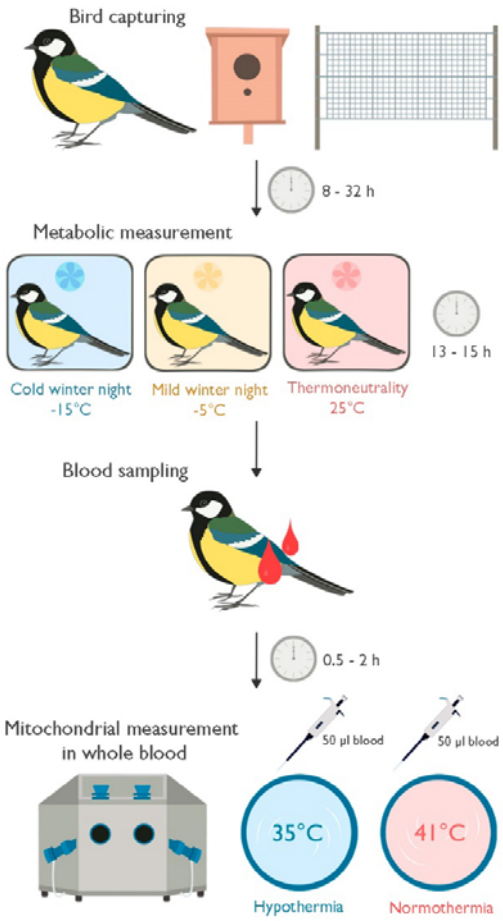

Figure 1. Overview of the experimental design and timeline of events when studying the effect of thermal state (normothermia or hypothermia) on functional properties of mitochondria in avian blood cells. Studies were performed using wild great tits (*Parus major*) that wintered outside the city of Lund in southernmost Sweden.

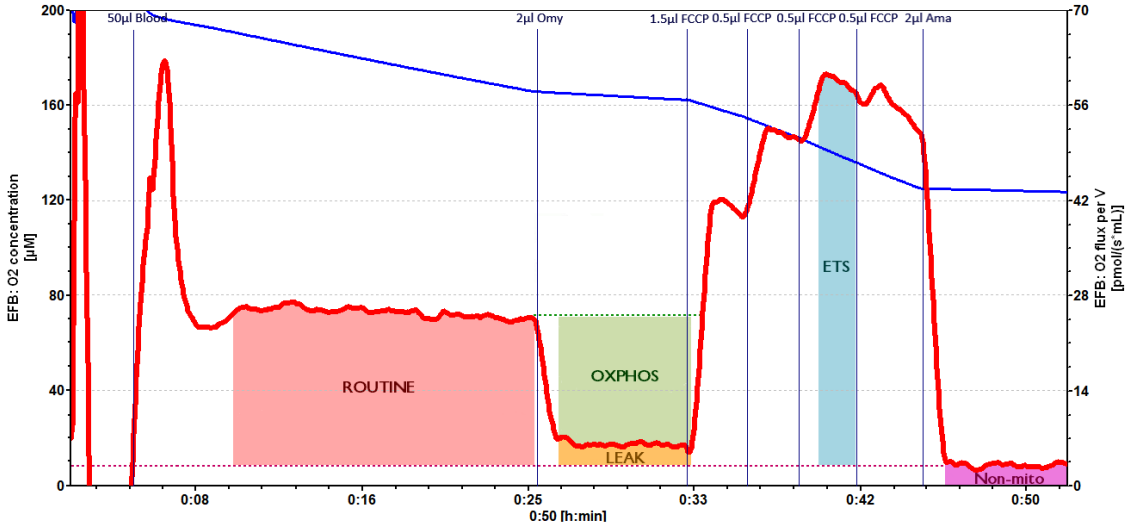

Figure 2. Representative trace of mitochondrial respiration in whole blood of a winter-adapted great tit (*Parus major*, individual 2KV07141) when measured at a normothermic daytime bird body temperature (41 °C). The blue line shows oxygen (O_2) concentration in the test chamber, and the red line shows O_2 consumption by the mitochondria on endogenous substrates (ROUTINE), after the addition of ATP synthase inhibitor oligomycin (Omy) (OXPHOS), when maximally stimulated by the mitochondrial uncoupler FCCP (carbonyl cyanide-p-trifluoro-methoxyphenyl-hydrazone) (ETS), and after inhibition by the cytochrome c reductase inhibitor antimycin A (Ama). LEAK refers to the part of respiration devoted to compensating for protons escaping over the inner mitochondrial membrane. "Non-mito" refers to non-mitochondrial respiration, i.e., oxygen consumption by the sample (such as binding of O_2 by haemoglobin) that is not due to mitochondrial use. Respiration states are defined in Table 1 and were superimposed on the raw data graph for clarity.

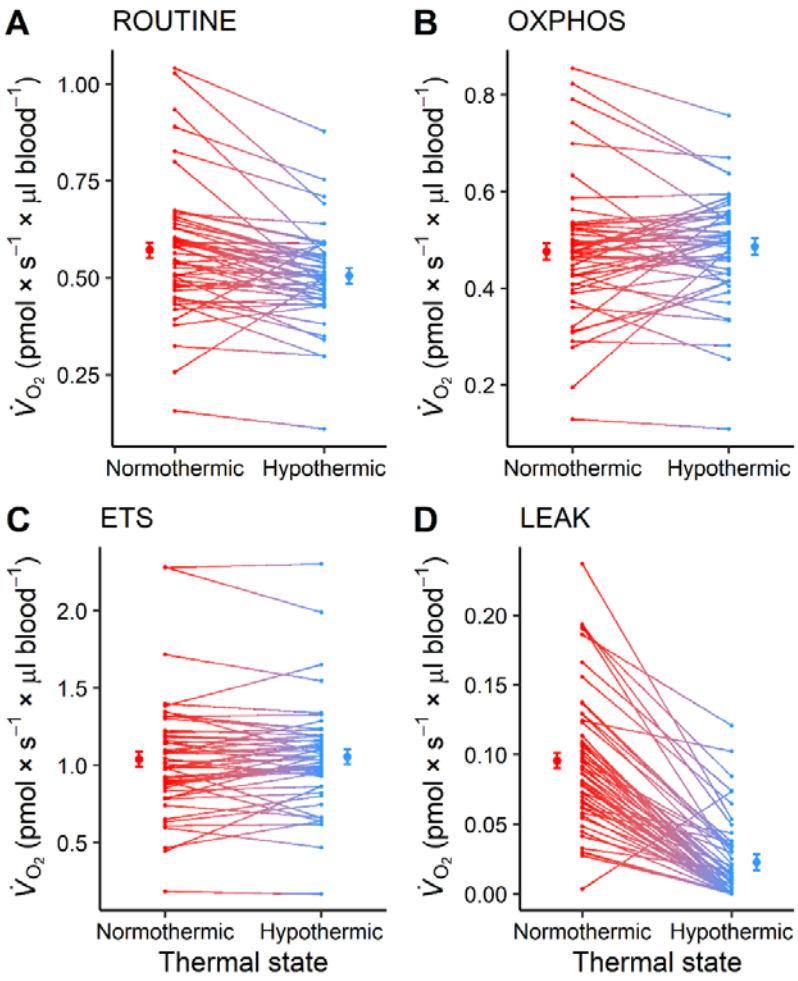

Figure 3. Mitochondrial respiration in whole blood of winter-adapted, wild, great tits (*Parus major*) at a representative daytime normothermic body temperature (41 °C) and a representative night-time hypothermic body temperature (35 °C). The same individuals were measured at both thermal states. Solid plotting symbols with error bars represent raw data means ± s.e.m. Gradient lines show individual responses. Sample sizes and statistics are reported in Table 2. (A) baseline ("ROUTINE") oxygen consumption during oxidative phosphorylation. (B) oxygen consumption devoted to ATP production alone ("OXPHOS") (C) maximum working capacity of the electron transport system when uncoupled from ATP production ("ETS"). (D) the part of ROUTINE used to offset proton leak across the inner mitochondrial membrane ("LEAK") in different assay temperatures after previous exposure to thermoneutral, mild, or very cold nights.

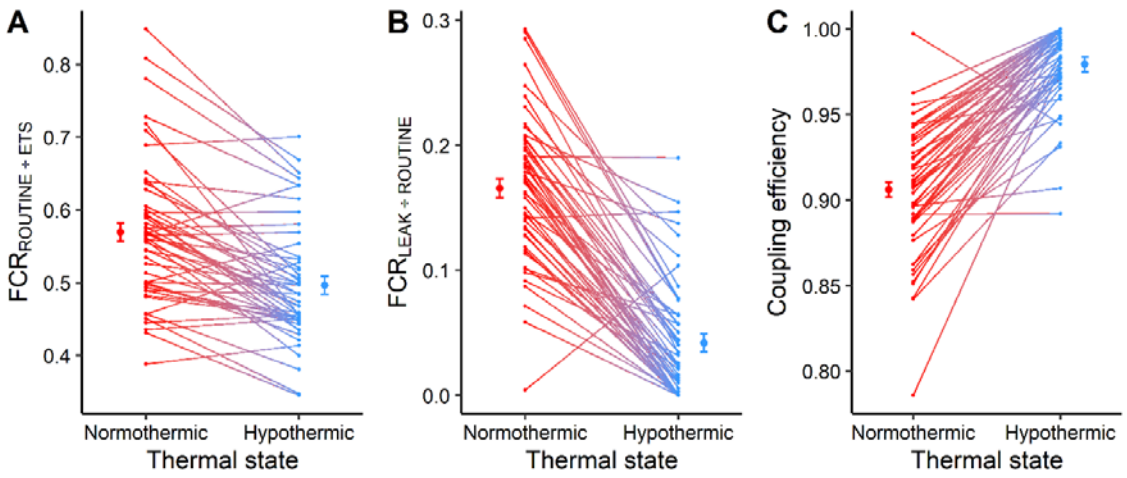

Figure 4. Flux control ratios (FCR) for mitochondrial respiration in great tit whole blood at a normothermic (41 ºC) and a hypothermic (35 ºC) thermal state. (A) An index of how intensively the mitochondria were respiring under endogenous conditions (ROUTINE) relative to their maximum working capacity (i.e., ETS). (B) The fraction of endogenous (ROUTINE) respiration that is used to compensate for LEAK respiration. (C) Coupling efficiency of electron transport to ATP production under maximally stimulated working capacity, i.e., the fraction of maximal respiration that is directed towards oxidative phosphorylation. Samples were collected from wild birds and the same individual was measured in both thermal states. Solid plotting symbols with error bars represent raw data means ± s.e.m. Gradient lines show individual responses. Sample sizes and statistics are reported in Table 2.