Plasticity of mitochondrial function restores fuel production during rest-phase hypothermia

Authors: Carmen C. García-Díaz[1], Imen Chamkha[2], Eskil Elmér[2], Andreas Nord[1,*]

[1] Lund University, Department of Biology, Section for Evolutionary Ecology, Sölvegatan 37, SE-223 62 Lund, Sweden

[2] Lund University, Department of Clinical Sciences, Mitochondrial Medicine, Sölvegatan 17, SE-221 84, Lund, Sweden

* Author for correspondence: AN, e-mail: andreas.nord@biol.lu.se

ORCID numbers:

CCGD: 0000-0002-7827-8104

IM: 0000-0002-6919-585X

EE: 0000-0001-7901-1826

AN: 0000-0001-6170-689X

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 animals downregulate body temperature by several degrees to save energy when sleeping (rest-phase hypothermia). Surprisingly, small birds that winter at high latitude have comparatively limited capacity for hypothermia and so pay large energy costs to stay warm during cold nights. Fuel for this is provided by mitochondria as adenosine triphosphate (ATP), but mitochondrial respiration can be lower during hypothermia because of the temperature-dependence of biological processes. Thus, there could be a conflict between increased organismal fuel demand and a lower mitochondrial capacity to provide it during hypothermia. We studied this enigma in blood cell mitochondria of winter-adapted great tits (*Parus major*). Hypothermia reduced mitochondrial respiration by 14%. However, this did not affect respiration allocated to ATP production ("oxidative phosphorylation") because there was an associated reduction of non-phosphorylating ("leak") respiration, from 17% in normothermia to 4% in hypothermia. This increase in coupling efficiency meant that 96% of total respiration was used to produce ATP in hypothermia, compared to 83% in normothermia. Our study shows that short-term plasticity of mitochondrial function can safeguard fuel production at times where over cellular respiration is suppressed. This casts new light on the process by which small organism endure harsh winter cold.

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

INTRODUCTION

Endothermic animals, such as mammals and birds, use considerable amounts of energy to increase heat production or dissipation to regulate a high, and comparatively stable, body temperature across a wide range of air temperatures. This energy cost of thermoregulation can sometimes become prohibitively high, for example when food is scarce or when weather conditions deteriorate. Many small to medium-sized endotherms use rest-phase hypothermia, a regulated reduction of resting body temperature below normal levels, to mitigate such energetic challenges. This may reduce metabolic demands by upwards of 50% [1]. It follows that most animals using rest-phase hypothermia have high metabolic intensity and lose heat quickly and rely on high-energy or ephemeral food sources [2]. This fits the description of "the little bird in winter" [sensu 3], 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 during the cold season. Yet, most birds in northern forests, such as tits and chickadees, typically show daily variation in body temperature within 5 °C [e.g. 4, 5, 6] and generally do not exceed 7-8 °C even in severe cold [7-9, but see 10]. Theoretical models predict that even such moderate body temperature reduction can carry survival benefits [11, 12]. 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. 13].

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) [14]. 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 [15-18, e.g. 19]. 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. 20], causing 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; 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 [21]. This process, known as mitochondrial proton leak, constitutes some 20-25% of resting metabolic rate in endotherms [22, 23]. 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 [24-27]. 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 [28-30] [but see 31, 32]. Similar studies on endotherms suggests that while phosphorylating respiration is often actively suppressed in hypometabolic animals, non-phosphorylating (i.e., leak) respiration is sometimes reduced, sometimes unaltered, and sometimes increased [reviewed by 33].

Previous endotherm studies have largely been performed in mammals during hibernation or in deep torpor; thermal states characterised by substantial metabolic downregulation [1] beyond what is feasible in most birds [but see 34]. Hence, previous data are not easily extrapolated to the problems faced by the little bird in winter that 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 [4, 35]. 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 [36-38], over longer time periods in line with age-related metabolic senescence [39] and according to environmental context [18]. Importantly, blood cell respiration is correlated to that in skeletal muscle fibres and brain tissue in birds [40, 41] and, in mammals, there is a correlation between respiration of mononuclear blood cells and kidney and heart tissue [42]. Thus, mitochondrial measurement on avian blood cells has potential to inform on analogous responses in key tissues for heat production (skeletal muscle) and energy management (heart, kidney, brain), all of which show pronounced seasonal acclimatisation to winter conditions in small, northern birds [43]. If better coupled mitochondria serve as an adaptation to secure substrate production even when the overall energy expenditure of the cell is reduced, we expected that coupling should increase during hypothermia and in response to low night-time temperature.

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, Chamkha & Elmér [44]. 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") [45] 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 at 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 [14], 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 and three respiratory control ratios (i.e., ratios between the respiratory states) [46] 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) [47] 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 ($P > 0.05$) based on likelihood ratio tests [48]. Predicted values and standard errors (s.e.m.) were calculated using the emmeans function in the emmeans package [49].

RESULTS

A representative normothermic experiment is presented in Fig. S1. All details on the statistical tests are presented in Table S1.

The effect of thermal state on ROUTINE was similar in all night temperatures (i.e., assay temperature × night temperature: $P = 0.634$) and ROUTINE was not affected by night temperature ($P = 0.528$). However, ROUTINE was 14 % higher in normothermia (0.567±0.019 pmol O_2 s^{-1} μl^{-1}) than in hypothermia (0.499±0.019 pmol O_2 s^{-1} μl^{-1}) ($P < 0.001$) (Fig. 2A), i.e., endogenous respiration was slower during hypothermia. OXPHOS was not influenced by the interaction between thermal state and night temperature ($P = 0.961$) and was also not affected by thermal state ($P = 0.745$) or night temperature ($P = 0.415$) alone (Fig. 2B). This was also true for ETS (thermal state × night temperature: $P = 0.262$; thermal state: $P = 0.501$; night temperature: $P = 0.358$) (Fig. 2C). However, LEAK respiration dropped markedly in the colder tissue ($P < 0.0001$), being more than three times higher during normothermia (0.096±0.004 pmol O_2 s^{-1} μl^{-1}) compared to during hypothermia (0.023±0.004 pmol O_2 s^{-1} μl^{-1}) (Fig. 2D).

E-R control efficiency was lower during hypothermia ($P < 0.001$), meaning that a hypothermic bird had more mitochondrial reserve capacity compared to a normothermic bird

(0.570±0.012 and.496±0.012, of 1, respectively) (Fig. 3A). There was also a trend towards reduced respiratory reserve capacity (i.e., lower E-R control efficiency) with decreasing temperature of the preceding night ($P = 0.091$), being 13% higher after a warm (25 °C) than a very cold (-15 °C) night (Table S1). The transition to a hypothermic state improved the efficiency of oxidative phosphorylation (i.e., R-L control efficiency increased; $P < 0.001$) such that nearly all (96%) of endogenous respiration was used to produce ATP in hypothermia, compared to 83% in normothermia (Fig. 3B). In other words, LEAK accounted for nearly 17% of endogenous respiration during normothermia, but in hypothermia LEAK contributed only 4% to ROUTINE. E-L coupling efficiency (i.e., coupling of the ETS when maximal respiration was stimulated using FCCP) increased from 91% (0.906±0.004) in normothermia to 98% (0.97b±0.004) in hypothermia (Fig. 3C). Thus, the electron transport chain was tighter in the hypothermic state.

DISCUSSION

We tested whether plasticity of mitochondrial function in great tit blood cells could compensate for reduced cellular respiration rate during rest-phase hypothermia. Endogenous respiration ('ROUTINE') was lower when the cells were hypothermic (Fig. 2A). However, the phosphorylating part of respiration (OXPHOS; in which ATP is produced) remained constant during the transition from normo- to hypothermia (Fig. 2B). At the same time, non-phosphorylating (i.e., LEAK) respiration decreased threefold (Fig. 2D). As a result, hypothermia was associated with significant increases in both phosphorylating efficiency (i.e., R-L control efficiency was higher; Fig. 3B) and tightness of electron transport during hard work (i.e., E-L coupling efficiency was higher; Fig. 3C). In fact, nearly all respiration (96%) was directed towards ATP production during hypothermia, and the ETS was almost perfectly

coupled (0.98, of 1) in this thermal state. Non-uniformity of changes to respiration traits indicates that the mitochondrial response to hypothermia were actively regulated and not merely a consequence of thermal suppression of the ETS [cf. 50, 51]. Hence, our study indicates that plasticity of mitochondrial function may allow the little bird in winter to secure sufficient substrate delivery to meet thermoregulatory demands despite a general downregulation of cellular respiration during hypothermia.

Reduced LEAK and improved phosphorylating and coupling efficiency during hypothermia is in keeping with the hypothesis of biochemical compensation for thermal suppression of cell respiration at low body temperature. However, tighter coupling can potentially result in increased production of reactive oxygen species (ROS) as a by-product of electron transport [21, 52], 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. 53], which is related to many medical disorders [54]. This has given rise to the "uncoupling to survive" hypothesis, in which mitochondrial ROS are implicated in the aging process [55, 56]. It is interesting to speculate that a hypothermic bird might have to accept an oxidative cost to uphold ATP production. 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 [57]. However, the role of mitochondria as sources of cellular ROS has been contended [58-60] and empirical evidence suggests that the relationship between body temperature, ATP production and ROS generation is not straightforward [27, 61, 62]. In line with this, even though (pharmacological) uncoupling protects birds from cold-induced oxidative stress [63], the evidence for clear fitness effects thereof is equivocal [64, 65].

We found no strong effects of temperature of the preceding night on mitochondrial respiration traits and respiratory control ratios. This contrasts what has been recorded as part of seasonal acclimatisation of blood cell respiration in the same model system [18] and in response to cold exposure in other tissue types [15-17]. It is possible that the period of exposure (one night) and acute nature of the air temperature manipulation was too short or too stochastic to serve as a reliable environmental cue prompting a change to cellular respiratory function. Yet, we found evidence of a reduction in surplus respiratory capacity (i.e., lowering of E-R control efficiency) with decreasing night-time temperature (Table S1). These striking difference in short- and long-term responses to colder temperature suggests it would be interesting to study the temporal resolution of mitochondrial thermal acclimatisation in some detail.

This study provides new perspectives to our understanding of how cellular- and whole animal metabolism interact when the little bird in winter adapts to low temperatures. In this context, our work aligns with the body of evidence highlighting the utility of blood cell respirometry to gain minimally invasive insight into functional ecology, evolutionary physiology, and gerontology in birds [36, 37, 39, 66]. We conclude that, in the short term, blood cell mitochondria maintain phosphorylating respiration by reducing proton leak to increasing coupling efficiency and, hence, ATP production efficiency when body temperature decreases. These changes come at no expense of maximum capacity of the ETS because mitochondrial respiratory scope was larger during hypothermia (i.e., E-R control efficiency was higher, Fig. 3A). Yet. it is possible that rest-phase hypothermia may come at the cost of increased oxidative stress [cf. 57]. Future work should explore this hypothesis by direct measurement of ATP- and ROS production, and membrane potential, to conclude on the consequences of changes to the efficiency of phosphorylating respiration for redox balance in normo- and hypothermic birds. Several endotherm studies show that mitochondrial function in blood cells

correlates to that in tissues and organs with clearly defined roles in heat production and seasonal acclimatisation (see above), suggesting data presented here may be representative of cellular responses to hypothermia at organismal levels. Even so, it is important that future studies formally address the uniformity of this mitochondrial plasticity across tissues and organs. More broadly, it would be relevant to increase taxonomic and phenotypic coverage of these studies, spanning cold-hardy, non-hypothermic, birds [e.g. 67] to those that routinely use shallow [e.g. 68] or deep torpor [69-71].

ACKNOWLEDGEMENTS

The authors thank Stefan Nord for managing the Räften feeding stations by which birds for the study were caught. SN also assisted with fieldwork and provided miscellaneous technical support during the study.

FUNDING

This study was supported by the Birgit and Hellmuth Hertz Foundation (grant no. 2017-39034), The Royal Physiographic Society in Lund (grant no. 2019-41011), the Swedish Research Council (grant no. 2020-04686) and Stiftelsen Lunds Djurskyddsfond (all to AN).

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 are deposited in Figshare: 10.6084/m9.figshare.19902277

CONFLICT OF INTEREST

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

REFERENCES

1. Ruf T., Geiser F. 2015 Daily torpor and hibernation in birds and mammals. *Biol Rev* **90**(3), 891-926. (doi:10.1111/brv.12137).
2. Geiser F. 2021 *Ecological physiology of daily torpor and hibernation*. Cham, Switzerland, Springer Nature Switzerland AG; 317 p.
3. Brodin A. 2007 Theoretical models of adaptive energy management in small wintering birds. *Phil Trans R Soc B***362**(1486), 1857-1871.
4. Nilsson J.F., Nilsson J.-Å., Broggi J., Watson H. 2020 Predictability of food supply modulates nocturnal hypothermia in a small passerine. *Biol Lett* **16**(6), 20200133. (doi:doi:10.1098/rsbl.2020.0133).
5. Nord A., Nilsson J.F., Nilsson J.-Å. 2011 Nocturnal body temperature in wintering blue tits is affected by roost-site temperature and body reserves. *Oecologia* **167**(1), 21-25.
6. Nord A., Nilsson J.F., Sandell M.I., Nilsson J.-Å. 2009 Patterns and dynamics of rest-phase hypothermia in wild and captive blue tits during winter. *J Comp Physiol B* **179**(6), 737-745. (doi:10.1007/s00360-009-0357-1 [doi]).
7. Reinertsen R.E., Haftorn S. 1984 The effect of short-time fasting on metabolism and nocturnal hypothermia in the willow tit *Parus montanus*. *J Comp Physiol* **154**(1), 23-28.
8. Reinertsen R.E., Haftorn S. 1983 Nocturnal hypothermia and metabolism in the willow tit *Parus montanus* at 63 degrees N. *J Comp Physiol* **151**(2), 109-118.
9. Haftorn S. 1972 Hypothermia of tits in the Arctic winter. *Orn Scand* **3**(2), 153-166.
10. Cooper S.J., Gessaman J.A. 2005 Nocturnal hypothermia in seasonally acclimatized Mountain Chickadees and Juniper Titmice. *Condor* **107**(1), 151-155.
11. Brodin A., Nilsson J.-Å., Nord A. 2017 Adaptive temperature regulation in the little bird in winter: predictions from a stochastic dynamic programming model. *Oecologia* **185**(1), 43-54. (doi:10.1007/s00442-017-3923-3).

12. Welton N.J., Houston A.I., Ekman J., McNamara J.M. 2002 A dynamic model of hypothermia as an adaptive response by small birds to winter conditions. *Acta Biotheoretica* **50**(1), 39-56.

13. Andreasson F., Nord A., Nilsson J.-Å. 2020 Age differences in night-time metabolic rate and body temperature in a small passerine. *J Comp Physiol B* **190**(3), 349-359. (doi:10.1007/s00360-020-01266-5).

14. Nicholls D.G., Ferguson S.J. 2013 *Bioenergetics*. 4th ed. Cambridge, MA, USA, Academic Press.

15. Liknes E.T., Swanson D.L. 2011 Phenotypic flexibility in passerine birds: Seasonal variation of aerobic enzyme activities in skeletal muscle. *J Therm Biol* **36**(7), 430-436. (doi:http://dx.doi.org/10.1016/j.jtherbio.2011.07.011).

16. Wang Y., Shan S., Zhang H., Dong B., Zheng W., Liu J. 2019 Physiological and biochemical thermoregulatory responses in male Chinese hwameis to seasonal acclimatization: Phenotypic flexibility in a small passerine. *Zool Stud* **58**, e6-e6. (doi:10.6620/ZS.2019.58-06).

17. Zheng W.H., Li M., Liu J.-S., Shao S.-L., Xu X.-J. 2014 Seasonal variation of metabolic thermogenesis in Eurasian tree Sparrows (*Passer montanus*) over a latitudinal gradient. *Physiol Biochem Zool***87**(5), 704-718. (doi:doi:10.1086/676832).

18. Nord A., Metcalfe N.B., Page J.L., Huxtable A., McCafferty D.J., Dawson N.J. 2021 Avian red blood cell mitochondria produce more heat in winter than in autumn. *FASEB J* **35**(5), e21490. (doi:https://doi.org/10.1096/fj.202100107R).

19. Schaeffer P.J., Villarin J.J., Lindstedt S.L. 2003 Chronic Cold Exposure Increases Skeletal Muscle Oxidative Structure and Function in Monodelphis domestica, a Marsupial Lacking Brown Adipose Tissue. *Physiol Biochem Zool***76**(6), 877-887. (doi:10.1086/378916).

20. Wright K.P., Jr., Hull J.T., Czeisler C.A. 2002 Relationship between alertness, performance, and body temperature in humans. *Am J Physiol* **283**(6), R1370-1377. (doi:10.1152/ajpregu.00205.2002).
21. Divakaruni A.S., Brand M.D. 2011 The Regulation and Physiology of Mitochondrial Proton Leak. *Physiology* **26**(3), 192-205. (doi:10.1152/physiol.00046.2010).
22. Porter R.K., Brand M.D. 1995 Causes of differences in respiration rate of hepatocytes from mammals of different body mass. *Am J Physiol* **269**(5 Pt 2), R1213-1224. (doi:10.1152/ajpregu.1995.269.5.R1213).
23. Rolfe D.F., Brand M.D. 1996 Contribution of mitochondrial proton leak to skeletal muscle respiration and to standard metabolic rate. *Am J Physiol***271**(4 Pt 1), C1380-1389. (doi:10.1152/ajpcell.1996.271.4.C1380).
24. Roussel D., Marmillot V., Monternier P.-A., Bourguignon A., Toullec G., Romestaing C., Duchamp C. 2020 Skeletal muscle metabolism in sea-acclimatized king penguins. II. Improved efficiency of mitochondrial bioenergetics. *J Exp Biol* **223**(21), jeb233684. (doi:10.1242/jeb.233684).
25. Bourguignon A., Rameau A., Toullec G., Romestaing C., Roussel D. 2017 Increased mitochondrial energy efficiency in skeletal muscle after long-term fasting: its relevance to animal performance. *J Exp Biol* **220**(13), 2445-2451. (doi:10.1242/jeb.159087).
26. Monternier P.A., Marmillot V., Rouanet J.L., Roussel D. 2014 Mitochondrial phenotypic flexibility enhances energy savings during winter fast in king penguin chicks. *J Exp Biol* **217**(15), 2691-2697. (doi:10.1242/jeb.104505).
27. Boël M., Romestaing C., Duchamp C., Veyrunes F., Renaud S., Roussel D., Voituron Y. 2020 Improved mitochondrial coupling as a response to high mass-specific metabolic rate in extremely small mammals. *J Exp Biol* **223**(5), jeb215558. (doi:10.1242/jeb.215558).

28. Glanville E.J., Seebacher F. 2006 Compensation for environmental change by complementary shifts of thermal sensitivity and thermoregulatory behaviour in an ectotherm. *J Exp Biol* **209**(24), 4869-4877. (doi:10.1242/jeb.02585).
29. Seebacher F. 2005 A review of thermoregulation and physiological performance in reptiles: what is the role of phenotypic flexibility? *J Comp Physiol B* **175**(7), 453-461. (doi:10.1007/s00360-005-0010-6).
30. Bouchard P., Guderley H. 2003 Time course of the response of mitochondria from oxidative muscle during thermal acclimation of rainbow trout, *Oncorhynchus mykiss*. *J Exp Biol* **206**(Pt 19), 3455-3465. (doi:10.1242/jeb.00578).
31. St-Pierre J., Brand M.D., Boutilier R.G. 2000 The effect of metabolic depression on proton leak rate in mitochondria from hibernating frogs. *J Exp Biol* **203**(9), 1469-1476.
32. Bishop T., St-Pierre J., Brand M.D. 2002 Primary causes of decreased mitochondrial oxygen consumption during metabolic depression in snail cells. *Am J Physiol* **282**(2), R372-R382. (doi:10.1152/ajpregu.00401.2001).
33. Staples J.F., Brown J.C.L. 2008 Mitochondrial metabolism in hibernation and daily torpor: a review. *J Comp Physiol B* **178**(7), 811-827.
34. McKechnie A.E., Lovegrove B.G. 2002 Avian facultative hypothermic responses: A review. *Condor* **104**(4), 705-724.
35. Nord A., Chiriac S., Hasselquist D., Nilsson J.-Å. 2013 Endotoxin injection attenuates rest-phase hypothermia in wintering great tits through the onset of fever. *Funct Ecol* **27**(1), 236-244. (doi:10.1111/1365-2435.12003).
36. Casagrande S., Stier A., Monaghan P., Loveland J.L., Boner W., Lupi S., Trevisi R., Hau M. 2020 Increased glucocorticoid concentrations in early life cause mitochondrial inefficiency and short telomeres. *J Exp Biol* **223**(15), jeb222513. (doi:10.1242/jeb.222513).

37. Stier A., Bize P., Hsu B.-Y., Ruuskanen S. 2019 Plastic but repeatable: rapid adjustments of mitochondrial function and density during reproduction in a wild bird species. *Biol Lett* **15**(11), 20190536. (doi:doi:10.1098/rsbl.2019.0536).

38. Udino E., George J.M., McKenzie M., Pessato A., Crino L., Buchanan K.L., Mariette M.M. 2021 Prenatal acoustic programming of mitochondrial function for high temperatures in an arid-adapted bird. *Proc R Soc Lond B* **288**, 20211893. (doi:doi:10.1098/rspb.2021.1893).

39. Dawson N.J., Salmón P. 2020 Age-related increase in mitochondrial quantity may mitigate a decline in mitochondrial quality in red blood cells from zebra finches (*Taeniopygia guttata*). *Exp Gerontol* **133**, 110883. (doi:https://doi.org/10.1016/j.exger.2020.110883).

40. Stier A., Romestaing C., Schull Q., Lefol E., Robin J.-P., Roussel D., Bize P. 2017 How to measure mitochondrial function in birds using red blood cells: a case study in the king penguin and perspectives in ecology and evolution. *Meth Ecol Evol* **8**(10), 1172-1182. (doi:10.1111/2041-210x.12724).

41. Stier A., Monaghan P., Metcalfe N.B. 2022 Experimental demonstration of prenatal programming of mitochondrial aerobic metabolism lasting until adulthood. *Proc R Soc Lond B* **289**(1970), 20212679. (doi:doi:10.1098/rspb.2021.2679).

42. Karamercan M.A., Weiss S.L., Villarroel J.P., Guan Y., Werlin E., Figueredo R., Becker L.B., Sims C. 2013 Can peripheral blood mononuclear cells be used as a proxy for mitochondrial dysfunction in vital organs during hemorrhagic shock and resuscitation? *Shock* **40**(6), 476-484. (doi:10.1097/shk.0000000000000026).

43. Petit M., Lewden A., Vézina F. 2014 How does flexibility in body composition relate to seasonal changes in metabolic performance in a small passerine wintering at northern latitude? *Physiol Biochem Zool***87**(4), 539-549. (doi:10.1086/676669).

44. Nord A., Chamkha I., Elmér E. 2022 A whole blood approach improves speed and accuracy when measuring mitochondrial respiration in avian hematocytes. *bioRxiv*, 2022.2005.2025.493398. (doi:10.1101/2022.05.25.493398).
45. Prinzinger R., Pressmar A., Schleucher E. 1991 Body temperature in birds. *Comp Biochem Physiol A* **99**(4), 499-506.
46. Gnaiger E. 2020 Mitochondrial pathways and respiratory control: An Introduction to OXPHOS Analysis. 5th ed.
47. Bates D., Maechler M., Bolker B., Walker S. 2015 Fitting linear mixed-effects models using lme4. *J Stat Soft* **67**, 1-48.
48. Zuur A.F., Ieno E.N., Walker N.J., Saveliev A.A., Smith G.M. 2009 *Mixed effects models and extensions in ecology with R*. New York, Springer.
49. Lenth R.V. 2019 emmeans: Estimated marginal means, aka least-squares means. R package version 1.3.3. https://CRAN.R-project.org/package=emmeans.
50. Frye L.D., Edidin M. 1970 The Rapid Intermixing of Cell Surface Antigens After Formation of Mouse-Human Heterokaryons. *J Cell Sci* **7**(2), 319-335. (doi:10.1242/jcs.7.2.319).
51. Blicher A., Wodzinska K., Fidorra M., Winterhalter M., Heimburg T. 2009 The temperature dependence of lipid membrane permeability, its quantized nature, and the influence of anesthetics. *Biophys J* **96**(11), 4581-4591. (doi:10.1016/j.bpj.2009.01.062).
52. Demine S., Renard P., Arnould T. 2019 Mitochondrial uncoupling: A key controller of biological processes in physiology and diseases. *Cells* **8**(8), 795. (doi:10.3390/cells8080795).
53. Brand M.D., Buckingham J.A., Esteves T.C., Green K., Lambert A.J., Miwa S., Murphy M.P., Pakay J.L., Talbot D.A., Echtay K.S. 2004 Mitochondrial superoxide and aging:

uncoupling-protein activity and superoxide production. *Biochem Soc Symp* (71), 203-213. (doi:10.1042/bss0710203).

54. Grasemann H., Holguin F. 2021 Oxidative stress and obesity-related asthma. *Paed Resp Rev* **37**, 18-21. (doi:10.1016/j.prrv.2020.05.004).
55. Brand M.D. 2000 Uncoupling to survive? The role of mitochondrial inefficiency in ageing. *Exp Gerontol* **35**(6-7), 811-820. (doi:10.1016/s0531-5565(00)00135-2).
56. Speakman J.R., Talbot D.A., Selman C., Snart S., McLaren J.S., Redman P., Krol E., Jackson D.M., Johnson M.S., Brand M.D. 2004 Uncoupled and surviving: individual mice with high metabolism have greater mitochondrial uncoupling and live longer. *Aging Cell* **3**(3), 87-95. (doi:10.1111/j.1474-9728.2004.00097.x).
57. Zagkle E., Grosiak M., Bauchinger U., Sadowska E.T. 2020 Rest-phase hypothermia reveals a link between aging and oxidative stress: A novel hypothesis. *Front Physiol* **11**, 575060. (doi:10.3389/fphys.2020.575060).
58. Zhang Y., Wong H.S. 2021 Are mitochondria the main contributor of reactive oxygen species in cells? *J Exp Biol* **224**(5), eb221606. (doi:10.1242/jeb.221606).
59. Mookerjee S.A., Divakaruni A.S., Jastroch M., Brand M.D. 2010 Mitochondrial uncoupling and lifespan. *Mech Ageing Devel* **131**(7-8), 463-472. (doi:10.1016/j.mad.2010.03.010).
60. Brown G.C., Borutaite V. 2012 There is no evidence that mitochondria are the main source of reactive oxygen species in mammalian cells. *Mitochondrion* **12**(1), 1-4. (doi:https://doi.org/10.1016/j.mito.2011.02.001).
61. Roussel D., Voituron Y. 2020 Mitochondrial costs of being hot: Effects of acute thermal change on liver bioenergetics in toads (*Bufo bufo*). *Front Physiol* **11**, 153. (doi:10.3389/fphys.2020.00153).

62. Boël M., Veyrunes F., Durieux A.-C., Freyssenet D., Voituron Y., Roussel D. 2022 Does high mitochondrial efficiency carry an oxidative cost? The case of the African pygmy mouse (*Mus mattheyi*). *Comp Biochem Physiol A* **264**, 111111. (doi:https://doi.org/10.1016/j.cbpa.2021.111111).

63. Stier A., Massemin S., Criscuolo F. 2014 Chronic mitochondrial uncoupling treatment prevents acute cold-induced oxidative stress in birds. *J Comp Physiol B* **184**(8), 1021-1029. (doi:10.1007/s00360-014-0856-6).

64. Stier A., Bize P., Roussel D., Schull Q., Massemin S., Criscuolo F. 2014 Mitochondrial uncoupling as a regulator of life-history trajectories in birds: an experimental study in the zebra finch. *J Exp Biol* **217**(19), 3579-3589. (doi:10.1242/jeb.103945).

65. Stier A., Bize P., Massemin S., Criscuolo F. 2021 Long-term intake of the illegal diet pill DNP reduces lifespan in a captive bird model. *Comp Biochem Physiol C* **242**, 108944. (doi:https://doi.org/10.1016/j.cbpc.2020.108944).

66. Stier A., Bize P., Schull Q., Zoll J., Singh F., Geny B., Gros F., Royer C., Massemin S., Criscuolo F. 2013 Avian erythrocytes have functional mitochondria, opening novel perspectives for birds as animal models in the study of ageing. *Frontiers in Zoology* **10**(1), 33. (doi:10.1186/1742-9994-10-33).

67. Nord A., Folkow L.P. 2018 Seasonal variation in the thermal responses to changing environmental temperature in the world's northernmost land bird. *J Exp Biol* **221**(1), jeb171124. (doi:10.1242/jeb.171124).

68. Romano A.B., Hunt A., Welbergen J.A., Turbill C. 2019 Nocturnal torpor by superb fairy-wrens: a key mechanism for reducing winter daily energy expenditure. *Biol Lett* **15**(6), 20190211. (doi:doi:10.1098/rsbl.2019.0211).

69. Hiebert S.M. 1990 Energy costs and temporal organization of torpor in the rufous hummingbird (*Selasphorus rufus*). *Physiol Zool* **63**, 1082-1097.

70. McKechnie A.E., Ashdown R.A.M., Christian M.B., Brigham R.M. 2007 Torpor in an African caprimulgid, the freckled nightjar *Caprimulgus tristigma*. *J Avian Biol* **38**(3), 261-266.

71. Brigham R.M. 1992 Daily torpor in a free-ranging goatsucker, the common poorwill (*Phalaenoptilus nuttallii*). *Physiol Zool* **65**(2), 457-472. (doi:10.2307/30158263).

TABLES

Table 1. Calculation method and definition of mitochondrial respiration states and control ratios. All traits were derived based on changes in O_2 consumption rate in response to the addition of mitochondrial agonists and antagonists during the assay as described in the main text.

Parameter	Calculation method	Definition
ROUTINE	$R_{Baseline}$ - $R_{Antimycin\ A}$	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} - $R_{Antimycin\ A}$	O_2 consumption when the electron transport system is operating at its maximum capacity.
LEAK	$R_{Oligomycin}$ - $R_{Antimycin\ A}$	O_2 consumption needed to compensate for proton leakiness of the mitochondria.
E-R control efficiency	1 - (ROUTINE / ETS)	Proportion of ETS maximum working capacity used under endogenous cellular conditions.
R-L control efficiency	1 - (LEAK / ROUTINE)	Proportion of endogenous respiration used for ATP production via oxidative phosphorylation.
E-L coupling efficiency	1 - (LEAK / ETS)	Tightness of electron transport during ATP production in a stimulated cellular state.

FIGURES

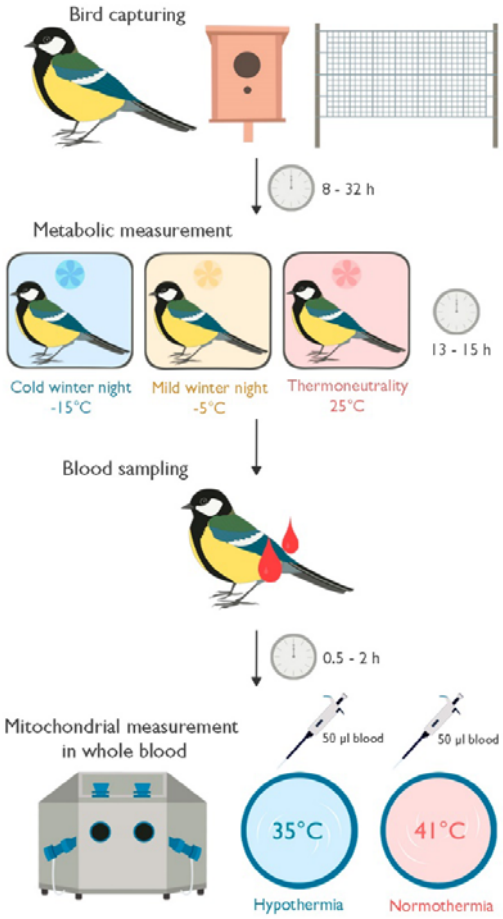

Figure 1. Overview of the experimental design and timeline of events when studying the effect of thermal state (normothermia or hypothermia) on functional properties of mitochondria in avian blood cells. Studies were performed using wild great tits (*Parus major*) that wintered outside the city of Lund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

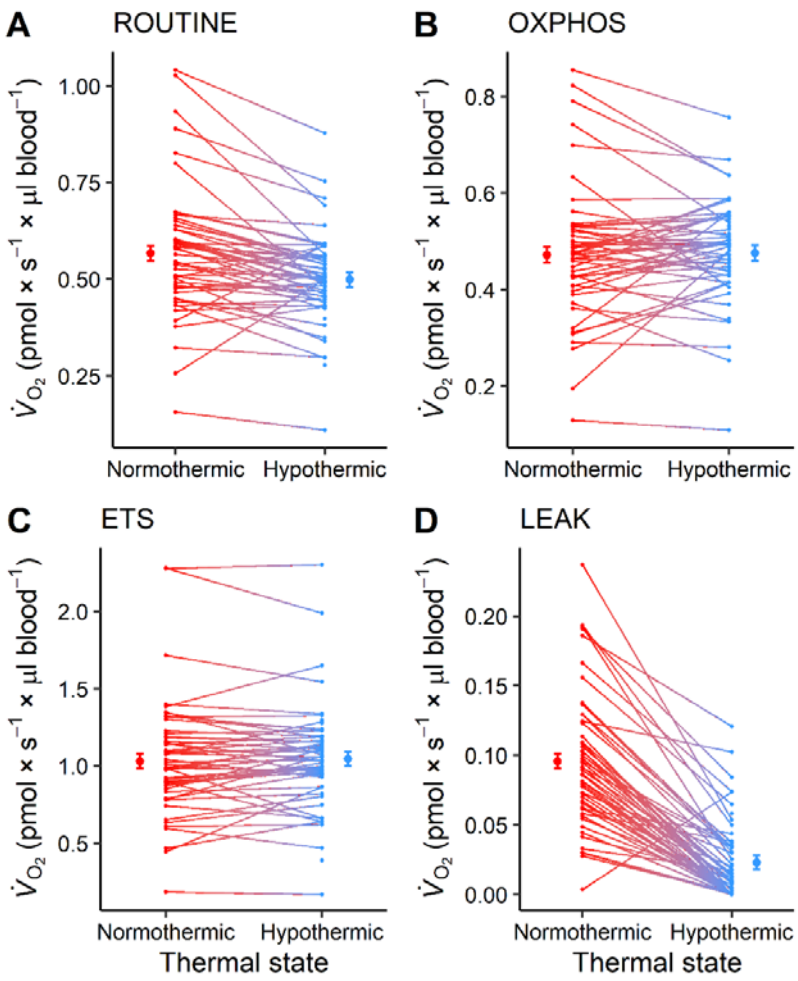

Figure 2. Mitochondrial respiration traits in whole blood of winter-adapted, wild, great tits (*Parus major*) at a representative daytime normothermic body temperature (41 °C) and a representative night-time hypothermic body temperature (35 °C). The same individuals were measured in both thermal states. Solid plotting symbols with error bars represent model-predicted means ± s.e.m. Gradient lines show individual responses. Sample sizes and statistics are reported in Table S1. (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 ("ETS"). (D) the part of ROUTINE used to offset proton leak across the inner mitochondrial membrane ("LEAK").

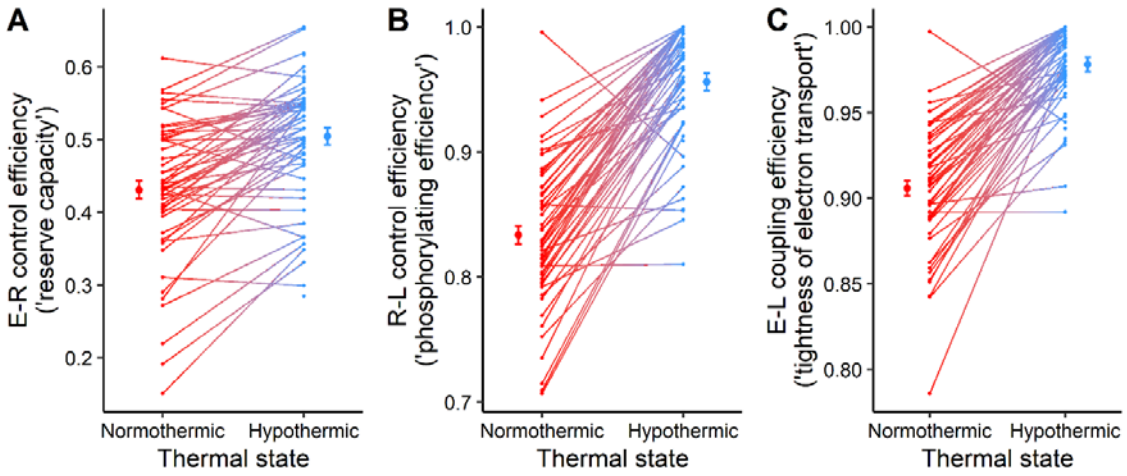

Figure 3. Control ratios for mitochondrial respiration in great tit whole blood in a normothermic (41 ºC) and a hypothermic (35 ºC) thermal state. (A) E-R control efficiency indicates how intensively the mitochondria were respiring under endogenous conditions (i.e., ROUTINE) relative to their maximum working capacity (i.e., ETS). For example, a value of 0.4 indicates that mitochondrial respiration can increase by 40% before reaching maximum. (B) The fraction of endogenous respiration that is used for oxidative phosphorylation where ATP is formed. (C) Coupling efficiency of electron transport when the ETS is stimulated to work at maximum capacity, where a value of 0 indicates a fully uncoupled system and a value of 1 a fully coupled system (i.e., where all respiration is towards phosphorylation). Samples were collected from wild birds and the same individual was measured in both thermal states. Solid plotting symbols with error bars represent model-predicted means ± s.e.m. Gradient lines show individual responses. Sample sizes and statistics are reported in Table S1.