© 2022. Published by The Company of Biologists Ltd | Journal of Experimental Biology (2022) 225, jeb242612. doi:10.1242/jeb.242612

RESEARCH ARTICLE

How does mitochondrial function relate to thermogenic capacity and basal metabolic rate in small birds?

Myriam S. Milbergue[1,2], François Vézina[1,2,3], Véronique Desrosiers and Pierre U. Blier[1,2,4,*]

ABSTRACT

We investigated the role of mitochondrial function in the avian thermoregulatory response to a cold environment. Using black-capped chickadees (*Poecile atricapillus*) acclimated to cold (−10°C) and thermoneutral (27°C) temperatures, we expected to observe an upregulation of pectoralis muscle and liver respiratory capacity that would be visible in mitochondrial adjustments in cold-acclimated birds. We also predicted that these adjustments would correlate with thermogenic capacity (M_{sum}) and basal metabolic rate (BMR). Using tissue high-resolution respirometry, mitochondrial performance was measured as respiration rate triggered by proton leak and the activity of complex I ($OXPHOS_{CI}$) and complex I+II ($OXPHOS_{CI+CII}$) in the liver and pectoralis muscle. The activity of citrate synthase (CS) and cytochrome *c* oxidase (CCO) was also used as a marker of mitochondrial density. We found 20% higher total CS activity in the whole pectoralis muscle and 39% higher total CCO activity in the whole liver of cold-acclimated chickadees relative to that of birds kept at thermoneutrality. This indicates that cold acclimation increased overall aerobic capacity of these tissues. M_{sum} correlated positively with mitochondrial proton leak in the muscle of cold-acclimated birds while BMR correlated with $OXPHOS_{CI}$ in the liver with a pattern that differed between treatments. Consequently, this study revealed a divergence in mitochondrial metabolism between thermal acclimation states in birds. Some functions of the mitochondria covary with thermogenic capacity and basal maintenance costs in patterns that are dependent on temperature and body mass.

KEY WORDS: Cold acclimation, Mitochondria, M_{sum}, Metabolic intensity, Oxidative phosphorylation, LEAK, Birds

INTRODUCTION

Upregulation of cold endurance in small avian species wintering at northern latitudes is typically associated with an enlargement of the skeletal muscles, heart and digestive organs and with elevated physiological maintenance costs (basal metabolic rate, BMR) and maximal heat production capacity (summit metabolic rate, M_{sum}; Swanson and Liknes, 2006; McKechnie, 2008; McKechnie and Swanson, 2010; Swanson and Vézina, 2015; McKechnie et al., 2015; Swanson et al., 2017). As shivering appears to be the main thermogenic mechanism used by birds living in cold environments (Saarela et al., 1995; Hohtola et al., 1998; Marjoniemi and Hohtola, 1999; Hohtola, 2004), the seasonal increase in muscle mass is often interpreted as the driver of M_{sum} variation. This interpretation is supported by a number of studies showing that seasonal elevations of M_{sum} are indeed associated with parallel increases of muscle mass (O'Connor, 1995; Cooper, 2002; Vézina et al., 2011; Swanson et al., 2013, 2014a; Petit et al., 2014; Swanson and Vézina, 2015) and by correlations between muscle mass and M_{sum} (Vézina et al., 2011; Swanson et al., 2013; Petit et al., 2014; Barceló et al., 2017; Milbergue et al., 2018). However, recent studies suggest that metabolic intensity, the energy consumed per unit mass of tissue (Vézina and Williams, 2005; Swanson, 2010), may also play a significant role in explaining the variation in both BMR and M_{sum} (Vézina and Williams, 2005; Vézina et al., 2011; Swanson et al., 2014a,b; Stager et al., 2015; Milbergue et al., 2018), and that mitochondrial function could be a key player in this phenomenon (Roussel et al., 1998; Zheng et al., 2008, 2014; Teulier et al., 2010; Stager et al., 2015; Vézina et al., 2017).

Oxidative phosphorylation involves the mitochondrial electron transport system (ETS), a set of protein complexes that transfers electrons and creates a proton-driving force from either side of the mitochondrial inner membrane. This force is used to generate ATP, an energy-carrying molecule supporting most cellular processes, including muscle contraction (Nicholls and Ferguson, 1992; Silva, 2003; Divakaruni and Brand, 2011). Higher oxidative phosphorylation capacity could thus allow higher muscle contractile activity such as shivering, as ATP is required for actin–myosin-ATPase and Ca^{2+}-ATPase activities. As oxidative respiration is not 100% efficient in transferring nutrient energy to ATP (Rolfe and Brown, 1997; Hill et al., 2004), heat is generated as a by-product and this production can be magnified by a proton leak through the inner mitochondrial membrane (Brand et al., 2005; Divakaruni and Brand, 2011). In mammals, mitochondrial proton leak is estimated to represent 20–30% of basal metabolism (Rolfe and Brand, 1997a, b; Rolfe et al., 1999; Divakaruni and Brand, 2011) and is thought to be involved in thermogenesis in response to cold (Lowell and Spiegelman, 2000; Mozo et al., 2005). In birds, the influence of proton leak on thermogenesis remains unclear. Nevertheless, cold environments can induce higher mitochondrial respiratory capacity in birds. This is demonstrated by increases in the activity of citrate synthase (CS), a regulatory enzyme of the Krebs cycle, and of cytochrome *c* oxidase (CCO), the last complex of the ETS, in the liver, kidneys and muscles of small passerines and shorebirds (Zheng et al., 2008; Liknes and Swanson, 2011; Vézina et al., 2017). Increases of LEAK respiration following exhaustion of ADP (state 4 respiration) is also observed in cold-acclimatized or -acclimated Chinese bulbul (*Pycnonotus sinensis*), tree sparrow (*Passer montanus*) and muscovy duckling (*Cairina moschata*) (Roussel et al., 1998; Zheng et al., 2008, 2014; Teulier et al., 2010). The involvement of these cellular adjustments in the whole-organism metabolic response to cold still needs clarification. Indeed, although some studies have investigated the relationship

[1]Département de Biologie, Chimie et Géographie, Université du Québec à Rimouski, 300 Allée des Ursulines, Rimouski, QC, Canada, G5L 3A1. [2]Groupe de Recherche sur les Environnements Nordique BORÉAS. [3]Centre d'Études Nordiques. [4]Centre de la Science de la Biodiversité du Québec, Canada.

*Author for correspondence (pierre_blier@uqar.ca)

M.S.M., 0000-0003-0168-6684; F.V., 0000-0002-9661-241X; P.U.B., 0000-0003-1881-2421

Received 4 August 2021; Accepted 24 January 2022

between M_{sum} variation and mitochondrial properties such as lipid transport across membranes (Stager et al., 2015; Vézina et al., 2017; Zhang et al., 2017) and CS activity (Swanson et al., 2013, 2014b; Stager et al., 2015; Vézina et al., 2017; Zhang et al., 2017), only very few studies, to our knowledge, have investigated the potential influence of the proton leak and oxidative phosphorylation capacity on thermogenesis in birds.

Mitochondrial respiratory capacity can be adjusted in two different ways. Firstly, through an increase in mitochondrial density (change of total mitochondrial volume and/or membrane surface) and therefore in enzyme content and maximal enzymatic activity. Such an increase typically enhances aerobic capacity (Johnston et al., 1998) and is known to occur both in mammals and birds in association with winter or experimental cold conditions (Mathieu-Costello et al., 1998; Nisoli et al., 2003; Zheng et al., 2008, 2014). Secondly, changes of respiratory capacity could also occur alternatively or concomitantly through adjustment of mitochondrial structure and function such as variation in the activity of certain mitochondrial enzymes, cristae density or the phospholipid composition of mitochondrial membrane (St-Pierre et al., 1998; Nielsen et al., 2017). Because of the respective location of CS and COO in the mitochondria (matrix and inner membrane), their activity can be used as a proxy of mitochondrial volume and surface density, respectively, and thus as a proxy of tissue mitochondrial content (Picard et al., 2011, 2012; Larsen et al., 2012; Munro et al., 2013), which allows evaluation of mitochondrial quantitative adjustments. Reporting mitochondrial respiration per unit CS and CCO partly controls for mitochondrial content and therefore allows changes in the mitochondrial phenotype to be studied. This approach has been used in fishes, rats, humans and bivalves (Salin et al., 2016; Picard et al., 2010, 2011; Rabøl et al., 2010; Munro et al., 2013) but, to our knowledge, it has not been used with birds.

We recently found that captive cold-acclimated (−10°C) black-capped chickadees (*Poecile atricapillus*) were maintaining a 20% higher M_{sum} than individuals kept at thermoneutrality (27°C), despite a comparable mass of pectoralis and total skeletal muscles (Milbergue et al., 2018), and suggested that this improved thermogenic capacity in the cold required an up-regulation of cell function. Here, we present a follow-up study where we document changes in mitochondrial respiratory function (oxidative phosphorylation and LEAK) in the liver and pectoralis muscles of these same birds with the aim of uncovering the potential mechanisms involved. If higher thermogenic capacity results from changes in mitochondrial function in cold-acclimated birds, we would expect to find higher oxidative phosphorylation and/or LEAK in pectoralis muscles in the cold and that these parameters would correlate positively with M_{sum}. As changes in metabolic intensity occurring with thermal acclimation can also influence energy consumption in a resting state, we also investigated potential relationships between mitochondrial function and BMR variation. In this case, we predict positive relationships between BMR and mitochondrial function (Zheng et al., 2008, 2013; Peña-Villalobos et al., 2014; Zhou et al., 2016), particularly LEAK respiration, as up to 30% of BMR variation is explained by LEAK in pectoralis muscle and liver of rats (Rolfe and Brown, 1997; Rolfe et al., 1999) and because significant correlations have been found between BMR and LEAK in birds (Zhou et al., 2016).

MATERIALS AND METHODS

Animals

Wild black-capped chickadees, *Poecile atricapillus* (Linnaeus 1766), were captured at the Forêt d'enseignement et de recherche Macpès (48°19N, 68°30W) and lac à l'Anguille (48°25N, 68°25W), both in eastern Quebec, Canada. They were then held in individual cages (39 cm×43 cm×31 cm) at the avian facilities of the Université du Québec à Rimouski under a constant photoperiod (10 h:14 h light:dark) and a temperature of −10°C for an average period of 2 months. Birds had *ad libitum* access to sunflower seeds, Mazuri small bird's maintenance diet (MAZURI® exotic animal nutrition) and water. They were fed with 0.20 g of living mealworms and 0.30 g of freshly thawed frozen crickets each day. They also received a supplement of electrolytes daily (Electrolytes plus, Vetoquinol N.-A. Inc., Princeville, QC, Canada) and vitamins once per week (Poly-tonine A® complex, Vetoquinol N.-A. Inc.) in their water. Acclimation started the day we separated the birds in two groups, with 24 individuals acclimated to −10°C (cold) and 25 individuals acclimated at the same time to 27°C (thermoneutral zone for this species; Rising and Hudson, 1974; Cooper and Swanson, 1994) for a minimum of 28 days before the metabolic measurements. All procedures respected the Canadian Council on Animal Care (CCAC) guidelines and were approved by the animal care committee of the Université du Québec à Rimouski (CPA-60-15-160). They were conducted under scientific and banding permits from Environment Canada–Canadian Wildlife Service.

BMR and M_{sum} measurement

Following thermal acclimation, we carried out metabolic rate measurements using the protocol and respirometry setup described by Milbergue et al. (2018) with measurements conducted on 4 birds per day (2 per trial). Briefly, each M_{sum} trial was conducted on two randomly chosen birds from the same temperature treatment and began approximately at 09:00 h and 12:30 h (alternating thermal treatments between measurements). Trials began by exposing the birds, in individual stainless steel metabolic chambers (volume 1350 ml), to temperatures declining by 3°C every 20 min in a 79% helium, 21% oxygen environment (flow rate 900 ml min^{-1}, start temperature 0°C for cold-acclimated birds and 10°C for birds from the thermoneutral treatment). Trials ended when birds became hypothermic, which was identifiable as a decline in oxygen consumption for several minutes and confirmed by body temperature measured immediately after taking birds out of their chamber (Milbergue et al., 2018). M_{sum} is the highest 10 min average O_2 consumption measured over the trial.

After M_{sum} measurement, birds were returned to their cage and had access to food and water until BMR measurement, which was conducted on all 4 birds starting at around 19:00 h (temperature 30°C, within the thermoneutral zone; Rising and Hudson, 1974; Cooper and Swanson, 1994). Trials ended the following morning (at approximately 07:30 h, as the duration of BMR trials was >12 h; all birds were post-absorptive at the time of BMR measurement). BMR was measured as the lowest 10 min average O_2 consumption value during this 12 h period, which was usually found towards the end of the night. For both M_{sum} and BMR, body mass was measured prior to and after measurement and the average was used in analyses. Birds were then returned to their cages.

Permeabilization of hepatocytes and muscles fibers

Birds were euthanized by decapitation and biopsies of the right pectoralis muscle and liver were sampled within 10 min of the bird's death and were weighed with a precision balance (Cole-Parmer Symmetry, PA-Series; 0.0001 g). These fresh biopsies were then immediately immersed in an ice-cold wash and preservation solution (in mmol l^{-1}: 2.77 CaK_2EGTA, 7.23 K_2EGTA, 6.56 $MgCl_2$, 20 taurine, 0.5 DTT, 50 potassium-methane sulfonate, 5.77 Na_2ATP, 15

Journal of Experimental Biology (2022) 225, jeb242612. doi:10.1242/jeb.242612

creatine phosphate and 20 imidazole, pH 7.1: solution I; Scott et al., 2009). Mechanical permeabilization of biopsies was performed in this ice-cold solution by dissection using two extra-sharp antimagnetic forceps to help the liquid penetrate the tissues. We then transferred muscles samples in a permeabilizing solution (solution I plus 50 µg ml^{-1} of saponin) that was stirred for 30 min (Pesta and Gnaiger, 2011). Liver biopsies were directly placed in a respiration solution (in mmol l^{-1}: 0.5 EGTA, 3 $MgCl_2$, 60 potassium lactobionate, 20 taurine, 10 KH_2PO_4, 20 Hepes, 110 sucrose and 1 g l^{-1} fatty acid-free BSA, pH 7.1: solution II; Kuznetsov et al., 2002) and stirred for 10 min to wash out endogenous adenine nucleotides and creatine. After the 30 min of permeabilization, muscles samples were also placed in this respiration solution and stirred for 10 min. After drying muscle and liver samples quickly on a soft paper, we weighed tissues using an analytical balance (Mettler Toledo; 0.0001 g). It should be noted here that preliminary tests were also conducted on isolated mitochondria but despite multiple attempts, we could not obtain mitochondria of sufficient quality, as expressed by respiratory control ratio (RCR) or respiration rate following cytochrome *c* addition, or an appropriate magnitude of signal to be able to ensure decent measurements. This was obviously not the case with permeabilized fibers. We also aimed to work in conditions as close as possible to a natural physiological state, and therefore the permeabilized tissues were more suitable for this.

Mitochondrial respiratory measurements

We inserted hepatocytes and muscles fibers (0.7–2.7 mg wet mass) in respiration chambers of a high-resolution respirometer (Oroboros Oxygraph-2k, Innsbruck, Austria), in 2 ml of respiration solution II kept at 35°C under continuous stirring. Glutamate (10 mmol l^{-1}) and malate (10 mmol l^{-1}) were injected into chambers to stimulate non-phosphorylating respiration (i.e. LEAK, mitochondrial respiration in the absence of ATP production). Then, stepwise additions of ADP (5 mmol l^{-1}) and succinate (10 mmol l^{-1}) were conducted to measure OXPHOS, which is the respiration rate induced by the activation of NADH-dehydrogenase (CI) and of succinate dehydrogenase (CII), which provides electrons for the ETS and supports ADP phosphorylation. This combination of substrates gave us the maximal respiration capacity in tissues. A stable rate of O_2 consumption was reached after 5–30 min between each addition of substrates. The activity of LEAK, $OXPHOS_{CI}$ and $OXPHOS_{CI+CII}$ is presented in pmol O_2 s^{-1} mg^{-1} of wet tissue. Total values (total capacity of the whole tissue for each mitochondrial parameter) were calculated by multiplying the parameter (expressed by unit of tissue mass) by the total mass of the corresponding organ (pectoral muscle or liver). Values of RCR correspond to the ratio between $OXPHOS_{CI}$ and LEAK.

CCO and CS activity assays in liver and pectoralis muscle homogenates

Part of the pectoralis muscle and liver biopsies was frozen in liquid N_2 less than 10 min after extraction and stored at −80°C. Later, the frozen tissues were weighed and chopped with a razor blade and then homogenized on ice in 9 volumes of homogenization buffer (in mmol l^{-1}: 2 EDTA, 100 Tris-HCl and 0.05% Tween-20, pH 7.5) using an Heidolph DIAX 900 (Sigma-Aldrich) blender for 3×10 s for pectoralis muscle and 2×10 s for liver, at 40% of full power. The homogenates were then centrifuged at 4000 ***g*** for 5 min in a refrigerated centrifuge (Eppendorf 5810R) to pellet contractile proteins and cellular debris and to recover the supernatant. Part of the homogenate supernatant was stored at −80°C for protein content assays performed later (see below).

CCO and CS activity was assayed according to Thibeault et al. (1997) with a Perkin Elmer spectrophotometer at 35°C. The CCO assay medium contained 100 mmol l^{-1} potassium phosphate, 100 µmol l^{-1} cytochrome *c*, which was reduced with 500 mmol l^{-1} dithiothreitol at pH 8.0 (final concentration: 4.5 mmol l^{-1}). Enzyme activity was calculated by following oxidation of reduced cytochrome *c* using an extinction coefficient of 29.5 ml cm^{-1} µmol^{-1} at 550 nm. The CS assay medium contained 100 mmol l^{-1} Tris, 2 mmol l^{-1} EDTA, 0.1 mmol l^{-1} 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB), 0.2 mmol l^{-1} acetyl coenzyme A (acetyl CoA) and 0.5 mmol l^{-1} oxaloacetic acid (OAA), at pH 8.0. Enzyme activity was calculated using an extinction coefficient of 13.6 ml cm^{-1} µmol^{-1} at 412 nm. All enzyme assays were run in quadruplicate and activity is presented in U mg^{-1} of tissue (where 1 U=1 µmol min^{-1}). These values give us an estimation of CS and CCO activity in the muscle and liver biopsies and were used to measure mitochondrial respiration rate.

Protein content quantification

Protein concentration of wet pectoralis muscle and liver tissues was determined using the bicinchoninic acid (BCA) method (Walker, 1994) with bovine serum albumin (Bio-Rad) as protein standard. All protein assays were run in triplicate with an incubation time of 30 min at 37°C.

Statistical analysis

Our analyses first tested whether thermal treatments (−10°C and 27° C) generated differences in mitochondrial function or content in the liver and pectoralis muscle. We therefore (1) investigated the effects of thermal treatments on mitochondrial density by comparing the tissue activity of CS and CCO between treatments. We also (2) studied how treatments affected mitochondrial respiratory capacity by testing for treatment effects on LEAK and OXPHOS (CI and CI+CII) respiration rate. We then (3) determined whether acclimation to low temperature generated phenotypic changes in mitochondria by testing for effects of thermal treatments on mitochondrial respiratory capacity (LEAK and OXPHOS) reported per unit of CS and CCO activity. These analyses were performed using ANCOVA models that included the effect of thermal treatment and the effect of body mass as a covariate to consider their effects on tissue metabolism (West et al., 2002; Krijgsveld et al., 2012). Models also included the interaction treatment×body mass. Furthermore, we (4) investigated the potential link between mitochondrial respiratory capacity and maximal heat production, measured as M_{sum}, and resting energy consumption, measured as BMR. This was done using separate ANCOVA models testing for the effects of each mitochondrial parameter (CS, CCO, LEAK, OXPHOS) on M_{sum} and BMR using values per mg or g of tissue and values per unit CS and CCO. The models included the effects of treatment, body mass and the interactions treatment×body mass and treatment×mitochondrial parameter.

Analyses were conducted using R Studio (3.3.1) and JMP Pro (12.0.1). In all models, we eliminated non-significant interactions and variables. Outlier values were discriminated by the Cook distance test and removed from analyses if necessary. Normality and homoscedasticity of model residuals were confirmed in all analyses using the Shapiro–Wilk test, Bartlett test and the 'par' function to visualize residuals distribution graphically (Murrell, 2005). The unexpected death of some of the birds and technical issues during measurements led to differing sample size among treatments and among mitochondrial parameters.

Journal of Experimental Biology (2022) 225, jeb242612. doi:10.1242/jeb.242612

Table 1. Effect of thermal treatment and body mass on CS and CCO activity

	Activity		Treatment		Body mass	
	Cold	Thermoneutral	*F* (d.f.)	*P*	*F* (d.f.)	*P*
Liver						
CS	13.8±1.1	13.7±1.0	0.09 (1,34)	0.8	2.9 (1,35)	0.09
CCO	17.3±1.1	15.9±1.0	0.6 (1,34)	0.4	1.3 (1,35)	0.3
CS_{total}	1.1±0.09	1.0±0.09	1.2 (1,24)	0.3	7.1 (1,25)	**0.01**
CCO_{total}	1.4±0.09	1.0±0.09	10.3 (1,24)	**<0.01**	3.4 (1,24)	0.07
Pectoralis						
CS	114.5±7.8	100.9±7.2	1.6(1,35)	0.2	0.6 (1,34)	0.4
CCO	6.6±0.7	5.8±0.6	0.7 (1,35)	0.4	0.2 (1,34)	0.6
CS_{total}	46.9±3.4	39.1±3.2	5.5 (1,33)	**0.02**	7.2 (1,33)	**0.01**
CCO_{total}	2.7±0.3	2.3±0.3	1.2 (1,34)	0.3	0.5 (1,33)	0.5

Citrate synthase (CS) and cytochrome *c* oxidase (COO) activity (mean±s.e.m.) is given as μmol min^{-1} g^{-1} tissue and as total (whole-organ) values in μmol min^{-1}. CS and CCO values for the whole organ were calculated by multiplying the CS and CCO activity by g of tissue with the value of dry lean mass of the pectoral and liver. Lean dry mass values were on average 0.4 g for pectoral and 0.07 g for liver (see Milbergue et al. 2018). Bold indicates significance.

RESULTS

Markers of mitochondrial density

There was no significant influence of thermal treatment on CS and CCO activity in both the liver and the pectoralis muscle when expressed per g of tissue (Table 1). When considering total enzymatic activity for the whole organ (activity per mg of tissue multiplied by the organ total mass), the total activity of CCO in the whole liver and total CS activity in the whole pectoralis muscle were 39% and 20% higher at −10°C than at 27°C, respectively (Table 1). Total CS activity was also significantly and positively correlated with body mass in the pectoralis muscle and liver (Table 1).

Mitochondrial respiration

Thermal treatment did not affect mitochondrial respiratory measurements of pectoralis muscles (all $P>0.25$; Table S1). In the liver, however, temperature did influence mitochondrial phenotype. We observed a marginally non-significant effect of thermal treatment on LEAK and $OXPHOS_{CI+CII}$ normalized by CCO, with cold-acclimated birds expressing lower values compared with birds maintained at thermoneutrality (Table 2). Our data showed that LEAK normalized by CS ($LEAK_{CS}$) in the liver declined with body mass in cold-acclimated individuals, whereas it remained stable when birds were acclimated to 27°C (Table 2, Fig. 1). Independent regression analysis showed that this relationship with body mass was significant for birds kept at −10°C (adjusted $r^2=0.46$, $n=13$, $P<0.01$; $P=0.4$ at 27°C). We also observed an interaction between treatment and body mass for liver $OXPHOS_{CI}$ respiration rate (Table 2). Mitochondrial respiration capacity ($OXPHOS_{CI}$ rate) tended to increase with body mass in warm-acclimated birds and to decrease with mass in cold-acclimated individuals, although independent regressions were not significant in any case (lowest *P*-value=0.26, r^2 range: 0.02–0.48). Collectively, these results suggest that body mass dependence of proton leak and respiration rate induced by NADH dehydrogenase, in the liver mitochondria, varies with thermal regime.

BMR, M_{sum} and mitochondrial activity

As observed previously (Milbergue et al., 2018), both thermogenic capacity and BMR were higher in cold-acclimated birds than in individuals kept at thermoneutrality. In this specific sample of birds, M_{sum} ($n=30$) and BMR ($n=34$) were 20.4% and 14.3% higher, respectively, in the cold- relative to the warm-acclimated birds when considering the effect of body mass (M_{sum}: $F_{1,27}=31.2$, $P<0.0001$, mass: $F_{1,27}=9.5$, $P<0.01$ no interaction; BMR: $F_{1,30}=13.6$, $P<0.001$, mass: $F_{1,30}=6.5$, $P<0.05$, interaction: $F_{1,30}=4.1$, $P=0.05$).

Analyses testing for relationships between M_{sum} and mitochondrial respiration in muscles and liver confirmed the effect of treatment and body mass on mitochondrial measurements in all models (no significant interaction treatment×body mass). None of the liver mitochondrial respiration measurements covaried with M_{sum} ($P>0.5$ in all cases), whereas in the muscles, once controlling for the effect of body mass ($F_{1,19}=9.4$, $P<0.01$), we found correlations between LEAK and M_{sum} that depended on temperature (LEAK: $F_{1,19}=12.7$,

Table 2. Effect of thermal treatment and body mass on mitochondrial LEAK, $OXPHOS_{CI}$, $OXPHOS_{CI+CII}$ and RCR in the liver

	Activity (pmol O_2 s^{-1})		Treatment		Body mass		Interaction	
	Cold	Thermoneutral	*F* (d.f.)	*P*	*F* (d.f.)	*P*	*F* (d.f.)	*P*
LEAK per mg tissue	28.5±3.4	33.5±2.9	0.95 (1,30)	0.3	0.00 (1,30)	1.0	–	–
$LEAK_{CS}$	202.8±31.5	251.1±27.2	1.34 (1,26)	0.3	0.35 (1,26)	0.6	6.0 (1,26)	**<0.05**
$LEAK_{CCO}$	160.4±33.0	245.2±27.3	3.9 (1,30)	0.06	0.96 (1,28)	0.3	–	–
$OXPHOS_{CI}$ per mg tissue	25.3±4.1	30.4±3.5	0.9 (1,28)	0.35	0.01 (1,28)	0.9	5.7 (1,28)	**<0.05**
$OXPHOS_{CI/CS}$	186.8±35.8	245.6±31.9	1.5 (1,25)	0.2	0.0 (1,25)	1.0	9.1 (1,25)	**<0.01**
$OXPHOS_{CI/CCO}$	149.5±34.4	218.6±29.8	2.3 (1,26)	0.1	0.73 (1,26)	0.4	7.6 (1,26)	**<0.05**
$OXPHOS_{CI+CII}$ per mg tissue	96.1±9.8	102.6±8.2	0.28 (1,30)	0.6	0.0 (1,30)	0.95	–	–
$OXPHOS_{CI+CII/CS}$	713.1±91.2	813.4±77.6	0.7 (1,29)	0.4	0.07 (1,27)	0.8	–	–
$OXPHOS_{CI+CII/CCO}$	509.0±85.2	725.3±70.5	3.8 (1,30)	0.06	0.78 (1,28)	0.4	–	–
RCR	1.78±0.21	1.87±0.18	0.1 (1,31)	0.76	0.3 (1.29)	0.6	0.02 (1,28)	0.9

Activity data (means±s.e.m.) are shown for mitochondrial LEAK, oxidative phosphorylation activity of complex I ($OXPHOS_{CI}$) and complex I+II ($OXPHOS_{CI+CII}$) [per mg tissue and normalized to citrate synthase (CS) and cytochrome *c* oxidase (COO)], and respiratory control ratio (RCR) under the different thermal acclimation treatments. Values were obtained using ANCOVA models that included the effect of thermal treatment and the effect of body mass as a covariate. Models also included the interaction treatment×body mass. Bold indicates significance.

Journal of Experimental Biology (2022) 225, jeb242612. doi:10.1242/jeb.242612

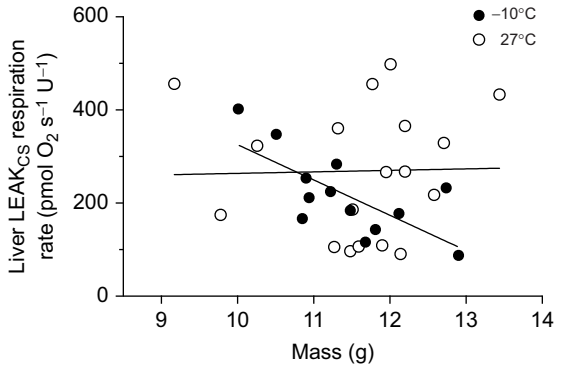

Fig. 1. Relationship between body mass and liver LEAK respiration rate normalized by citrate synthase (CS). Data are for the two acclimation treatments: cold (−10°C, *n*=13) and warm (thermoneutrality, 27°C, *n*=17).

P<0.01; $LEAK_{CS}$: $F_{1,16}$=9.2, P<0.01), with a strong positive covariation between M_{sum} and LEAK only in the cold (Fig. 2, LEAK: adjusted r^2=0.67, n=11, P<0.05; $LEAK_{CS}$: adjusted r^2=0.71, n=10, P<0.01). Independent regressions of M_{sum} and LEAK showed no clear relationships at thermoneutrality (P>0.2 in all cases).

We found no influence of pectoralis muscle mitochondrial content or respiratory capacity on BMR (only treatment and mass significantly affected BMR; see also Milbergue et al., 2018). In the liver, when statistically controlling for body mass ($F_{1,25}$=16.7, P<0.001), we found that BMR covaried with NADH dehydrogenase-dependent respiration rate ($OXPHOS_{CI}$), and that this influence depended on thermal treatment ($F_{1,25}$=8.9, P<0.01).

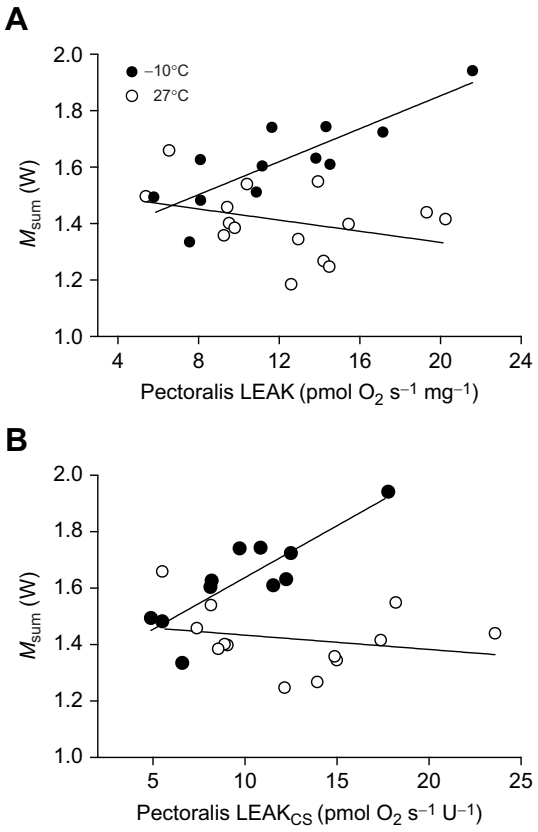

Fig. 2. Relationship between summit metabolic rate (M_{sum}) and LEAK in the pectoralis muscle. (A) LEAK per mg of tissue (−10°C: *n*=12; 27°C: *n*=15). (B) LEAK normalized by CS activity (−10°C, *n*=11; 27°C, *n*=13).

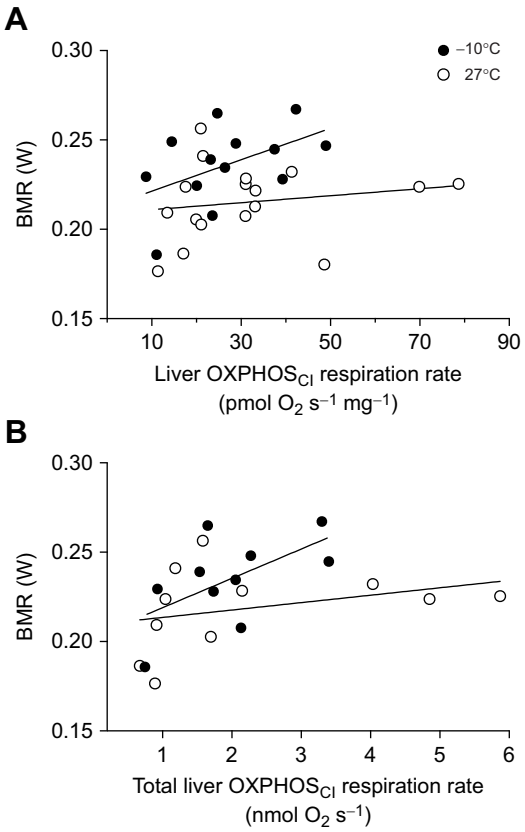

Fig. 3. Relationship between basal metabolic rate (BMR) and mitochondrial respiratory parameters in the liver. (A) Relationship between BMR and $OXPHOS_{CI}$ respiration rate per mg of tissue (−10°C, *n*=13; 27°C, *n*=17). (B) Relationship between BMR and total $OXPHOS_{CI}$ respiration rate (−10°C, *n*=10; 27°C, *n*=11).

This was observed in analyses considering both mass-specific and total liver $OXPHOS_{CI}$ respiration rate (Fig. 3). However, independent regression analyses showed that the only significant relationship was between total $OXPHOS_{CI}$ and BMR at −10°C (−10°C: r^2=0.39, n=10, P<0.05; 27°C: P=0.5). Although a similar pattern was observed when normalizing $OXPHOS_{CI}$ respiration rate by CS activity ($F_{1,22}$=7.3, P<0.05), no significant correlation was observed with BMR in independent regression analyses. Therefore, we found evidence that liver mitochondrial functions influence BMR, but only in cold-acclimated birds.

DISCUSSION

We predicted that, if an improvement in thermogenic capacity resulted mostly from changes in mitochondrial properties rather than muscle mass (Barceló et al., 2017; Milbergue et al., 2018), then oxidative phosphorylation and/or LEAK in pectoralis muscle should be higher at −10°C than at 27°C and should correlate positively with M_{sum}. We also expected that changes in metabolic intensity resulting from thermal acclimation could lead to correlations between BMR and both oxidative phosphorylation and LEAK in liver and pectoralis muscle. We found no differences in mitochondrial content in the liver and pectoralis muscles of cold- relative to warm-acclimated individuals when expressed per unit mass of tissue. However, we observed that mitochondrial phenotype varied with body mass in the liver of cold-acclimated birds. We also found that maximal oxidative phosphorylation, when mitochondria are fed at complex I, varied with BMR in these birds. However,

quantitative markers of mitochondrial respiratory capacity did not differ between treatments in the pectoralis muscle. Nevertheless, proton leakage measured in the pectoralis did correlate significantly with M_{sum} in cold-acclimated birds.

Markers of mitochondrial density

CS and CCO are regularly used as proxies for tissue maximal oxidative capacity and it has been shown that their activity increases in the liver and muscles of birds acclimated to cold wintering environments or cold experimental conditions (O'Connor, 1995; Dawson and Olson, 2003; Zheng et al., 2008, 2014; Liknes and Swanson, 2011; Peña-Villalobos et al., 2014; Vézina et al., 2017). In our study, there was no treatment effect on CS and CCO enzymatic activity when reported per g of tissue (Table 1). Our results therefore contrast with the observations of Liknes and Swanson (2011), who found notably higher (52%) CS activity per unit wet tissue mass in the pectoralis muscle of wintering chickadees, and of Zheng et al. (2008), who reported 37% higher CCO activity per mg of protein in the liver of winter-acclimatized Eurasian tree sparrow (*Passer montanus*) relative to birds measured in summer. The exact reasons for the discrepancies between these results and our study are not obvious but they might result from the difference in settings between the studies (experimental captives versus free living). Considering total organ activity, however, we found a 20% higher total CS activity in the pectoralis muscle and a 39% higher total CCO activity in the liver of cold-acclimated chickadees compared with birds kept at thermoneutrality (Table 1). These findings corroborate observations by Liknes and Swanson (2011), who reported a 62.5% higher total CS activity in the pectoralis muscles of black-capped chickadees wintering in South Dakota relative to their summer counterparts. Similarly, Zhou et al. (2016) reported higher total CCO activity in the liver of hwamei (*Garrulax canorus*) acclimated to 15°C relative to individuals kept at 35°C. Similar to our observations, Peña-Villalobos et al. (2014) also observed no differences in tissue mass-specific activity of CS or CCO between winter and summer in rufous-collared sparrow (*Zonotrichia capensis*) but found a 70% higher total CS activity in the flight muscles of cold-acclimated (15°C) compared with warm-acclimated (30°C) captive birds. Collectively, these results suggest that total aerobic capacity of both muscle and liver increase in birds acclimated to low temperatures. These results might seem counter-intuitive as muscle and liver mass, as well as mitochondrial capacity, were not significantly different between the two groups. This apparent discrepancy likely results from a multiplying effect, where differences in tissue mass and mitochondrial respiration rate between the two groups were not large enough to be detected as significant but amplified when multiplied with each other. Albeit being non-significant (P=0.17), Milbergue et al. (2018) did report 18% heavier livers (lean dry mass) and 5% heavier pectoralis muscles in cold-acclimated chickadees.

Mitochondrial respiration

Given that birds increased thermogenic capacity without changing muscle size (Milbergue et al., 2018), we expected to find higher oxidative phosphorylation and/or higher LEAK respiration in muscles of cold-acclimated birds as this could contribute to the heat production mechanism (Duchamp et al., 1999; Lowell and Spiegelman, 2010). Instead, our data revealed no effect of thermal treatment on pectoralis muscle mitochondrial respiration. This result came as a surprise, particularly for proton leak as it is suspected in endotherms that upregulating LEAK could operate to partially dissipate the proton-motive force in the mitochondria, thus leading to additional heat production (Duchamp et al., 1999; Divakaruni and Brand, 2011). Furthermore, several studies have reported higher pectoralis LEAK respiration in isolated mitochondria of cold-acclimated birds (Roussel et al., 1998; Teulier et al., 2010; Zheng et al., 2014; Zhou et al., 2016). Yet, it is not uncommon to find inconsistencies among such studies. For instance, muscle mitochondria of muscovy ducklings (*Cairina moschata*) had 38% higher state 3 respiration (oxidative phosphorylation) per mg of protein in individuals reared at 4°C compared with that in individuals reared at thermoneutrality (25°C; Teulier et al., 2010) and this contrasts with a previous study on the same species using the same thermal treatment, where no significant difference in muscle state 3 respiration was observed (Roussel et al., 1998). Another study on ducklings also reported no impact of temperature acclimation (11°C relative to 25°C) on oxidative phosphorylation and proton leak in the gastrocnemius muscle (Salin et al., 2015). In addition, Cheviron and Swanson (2017) observed no upregulation of genes putatively involved in non-shivering thermogenesis in winter- compared with summer-acclimatized American goldfinch and black- capped chickadee. Collectively, these findings suggest that the cellular adjustments observed in muscles of several species in association with cold (Roussel et al., 1998; Zheng et al., 2008, 2014; Teulier et al., 2010; Stager et al., 2015; Zhou et al., 2016) may be context specific or simply not part of an obligate adaptive response to cold environment in birds. Moreover, previous studies worked with isolated mitochondria and this technique can produce different results from permeabilized fibers as we used here (Kuznetsov et al., 2008; Korzeniewski, 2015; Mathers and Staples, 2015).

As for pectoral muscles, we expected to find higher oxidative phosphorylation capacity and/or higher LEAK respiration rate in the liver. Indeed, previous studies reported higher state 4 respiration in the liver of cold-acclimated Eurasian tree sparrow, Chinese bulbul and hwamei relative to values measured in individuals acclimated to summer or thermoneutrality (Zheng et al., 2008, 2014; Zhou et al., 2016). Mitochondrial function in the liver did seem to respond to temperature acclimation. Indeed, both the LEAK and rate of electron entry in the ETS normalized per CCO activity tended to be lower in cold-acclimated birds (LEAK/CCO 35%, $OXPHOS_{CI+CII}$/CCO 30%; Table 2) with the effect approaching significance for both variables (P=0.06). This suggests that the capacity to feed the ETS with electrons was reduced, relative to the capacity of CCO to reduce oxygen. Thus, in the liver of cold-acclimated birds, it seems that the electron pressure and the level of reduction in the ETS could be lower during non-phosphorylating and phosphorylating respiration. One would suspect that this reorganization could prevent the adverse impact of reactive oxygen species induced by increased mobilization of aerobic capacity. However, at this point we can only speculate on the adaptive function, if any, of these responses to cold acclimation and further investigation is required.

In the liver, both the proton leak (expressed per CS) and CI-dependent respiration rate ($OXPHOS_{CI}$) varied differently with body mass depending on thermal regime. The respiration rate tended to decrease with body mass in cold-acclimated birds (Fig. 1). Negative correlations have previously been reported in studies comparing species of various body sizes. For example, proton leak in the liver has been reported to negatively correlate with body mass in frogs (Roussel et al., 2015), mammals (Porter et al., 1996) and birds (Brand et al., 2003; Else et al., 2004). This correlation could potentially be associated with variation of fatty acyl composition in the mitochondrial membrane. Indeed, across a wide range of body mass in birds, from the 13 g zebra finches (*Taeniopygia guttata*) to the 35 kg emus (*Dromaius novaehollandiae*), proton conductance correlates positively with polyunsaturation and negatively with

monounsaturation of phospholipid fatty acyls (Brand et al., 2003; this is also found in mammals: Porter et al., 1996; Couture and Hulbert, 1995), supporting the idea that mitochondrial membranes act as a metabolic pacemaker (Hulbert and Else, 1999). Liver and muscle membranes from larger birds also have less polyunsaturated and more monounsaturated fatty acyl chains than those from smaller birds (Hulbert et al., 2002; Brand et al., 2003). Thus, although the variation in body mass was small among our birds, mitochondrial membranes of heavier birds at −10°C could have lower membrane proton conductance as a result of fatty acid profile (Hill et al., 2004). However, the exact cause of the apparent lack of mass effect at thermoneutrality is not obvious.

M_{sum}, BMR and mitochondrial activity

M_{sum} was strongly and positively correlated with mitochondrial proton leak in the pectoralis, but only for cold acclimated birds (Fig. 2). At first glance, the strong relationship found here suggests that cold-acclimated birds expressing higher LEAK values might have benefited from the increase in heat generated by less-efficient mitochondria. Yet, this result is counter-intuitive given that there was no treatment effect on LEAK per se (no significant differences between birds acclimated to −10°C and 27°C). This contribution of mitochondrial proton leak to maximal thermogenic capacity could thus be related to a trait that partly drives both the measured proton leak and the maximal respiration rate; for example, higher transmembrane potential or oxidative phosphorylation efficiency (ATP/O) or the effect of membrane fluidity on the catalytic capacity of membrane-embedded enzymatic complexes. However, this result will require further investigation.

In birds, BMR increases have been associated with elevated proton leak (Zheng et al., 2008, 2013, 2014), CS activity (Peña-Villalobos et al., 2014; Vézina et al., 2017) and oxidative phosphorylation (Zheng et al., 2013, 2014) in both liver and muscles. Our results indicated that BMR variation was partly related to oxidative phosphorylation capacity but only in the liver. They also showed that these relationships were temperature dependent. BMR increased with CI-dependent respiration rate but only in cold-acclimated birds (Fig. 3). We previously found that the relationship between body mass and BMR was uncoupled in cold-acclimated chickadees in such a way that the lightest birds had a BMR as high as that of the heaviest birds, whereas BMR was positively correlated with mass at thermoneutrality (Milbergue et al., 2018). We suggested that this phenomenon could be rooted in adjustments of metabolic intensity at the cellular level in cold-acclimated individuals. Interestingly, we observed here that in the cold, the lightest birds had high mitochondrial LEAK respiration rates in the liver (Fig. 1) and that, overall, cold-acclimated birds with high CI respiration rate also had high BMR (Fig. 3). The current finding is therefore consistent with our previous hypothesis. It suggests that mitochondrial adjustments in internal organs, such as the liver, might contribute to overall resting energy consumption, although this influence differs depending on the acclimation state of the birds. Changes in liver phenotype in association with cold acclimation have been noted in several bird species before. Reported changes are typically an increase of liver mass (Williams and Tieleman, 2000; Zheng et al., 2008, 2014; Petit et al., 2014; Barceló et al., 2017), which has been identified as a response to elevated cold-induced food consumption (Barceló et al., 2017), and which may significantly influence BMR variation (Chappell et al., 1999; Williams and Tieleman, 2000; Petit et al., 2014; Barceló et al., 2017). In our experiment, cold-acclimated chickadees consumed 44% more food on average and, although not significant, their livers were also 18% larger than those of individuals from the thermoneutral group (Milbergue et al., 2018).

Our study showed variation in quantitative and qualitative aspects of mitochondrial respiration between thermal acclimation states in birds. Some of the respiratory parameters co-varied with maximal thermogenic capacity and basal maintenance costs, depending on temperature and body mass. In cold-acclimated birds, M_{sum} was positively correlated with mitochondrial proton leak in the pectoralis muscle while BMR was positively correlated with oxidative phosphorylation capacity in the liver. This connection between LEAK or oxidative phosphorylation and whole-animal metabolic performance vanished in birds acclimated to 27°C. Although the exact mechanism explaining how birds can improve thermogenic capacity without changing muscle mass still needs clarification, our study suggests that this capacity is nevertheless associated with significant adjustments in muscle or liver mitochondrial machinery. Our study also demonstrated that the increase of thermogenic capacity in these birds was not simply a result of upregulating proton leak or oxidative phosphorylation, as often suggested. Consequently, it might also not be the result of an improvement of muscles fiber ATP production for shivering but this remains to be confirmed.

Acknowledgements

We are grateful to the Corporation de la Forêt d'Enseignement et de Recherche Macpès, who granted us access to the field facilities for capturing the birds used in this study. We thank Mikaël Jaffré, Aymeric Bodin and Gaël Lafenêtre for their help in capturing the birds as well as Karine Dubois for her help with dissections and organ processing. We thank Nicolas Pichaud, Daniel Munro and Felix Christen for their help and advice concerning mitochondrial processes. We are also grateful to Lyette Régimbald for the maintenance of captive birds and to Jonathan Coudé for his help with laboratory material. Alain Caron provided invaluable statistical advice.

Competing interests

The authors declare no competing or financial interests.

Author contributions

Conceptualization: M.S.M., F.V., P.U.B.; Methodology: M.S.M., F.V., V.D., P.U.B.; Formal analysis: M.S.M., F.V.; Investigation: M.S.M., V.D.; Resources: F.V., P.U.B.; Writing - original draft: M.S.M.; Writing - review & editing: F.V., P.U.B.; Visualization: M.S.M., F.V.; Supervision: F.V., V.D., P.U.B.; Funding acquisition: P.U.B.

Funding

This work was funded by a Team Research Project grant from the Fonds Québécois de la Recherche sur la Nature et les Technologies (FRQNT) and by Discovery grants from the Natural Sciences and Engineering Research Council of Canada (NSERC) to F.V. and P.U.B. It also benefited from a Leader Opportunity Fund award from the Canada Foundation for Innovation (CFI) to F.V.

References

Barceló, G., Love, O. P. and Vézina, F. (2017). Uncoupling basal and summit metabolic rates in white-throated sparrows: digestive demand drives maintenance costs, but changes in muscle mass are not needed to improve thermogenic capacity. *Physiol. Biochem. Zool.* **90**, 153-165. doi:10.1086/689290

Brand, M. D., Turner, N., Ocloo, A., Else, P. L. and Hulbert, A. J. (2003). Proton conductance and fatty acyl composition of liver mitochondria correlates with body mass in birds. *Biochem. J.* **376**, 741-748. doi:10.1042/bj20030984

Brand, M. D., Pakay, J. L., Ocloo, A., Kokoszka, J., Wallace, D. C., Brookes, P. S. and Cornwall, E. J. (2005). The basal proton conductance of mitochondria depends on adenine nucleotide translocase content. *Biochem. J.* **392**, 353-362. doi:10.1042/BJ20050890

Chappell, M. A., Bech, C. and Buttemer, W. A. (1999). The relationship of central and peripheral organ masses to aerobic performance variation in house sparrows. *J. Exp. Biol.* **202**, 2269-2279. doi:10.1242/jeb.202.17.2269

Cheviron, Z. A. and Swanson, D. L. (2017). Comparative transcriptomics of seasonal phenotypic flexibility in two North American songbirds. *Integr. Comp. Biol.* **57**, 1040-1054. doi:10.1093/icb/icx118

Cooper, S. J. (2002). Seasonal metabolic acclimatization in mountain chickadees and juniper titmice. *Physiol. Biochem. Zool.* **75**, 386-395. doi:10.1086/342256

Cooper, S. J. and Swanson, D. L. (1994). Seasonal acclimatization of thermoregulation in the black-capped chickadee. *Condor* **96**, 638-646. doi:10.2307/1369467

Couture, P. and Hulbert, A. J. (1995). Membrane fatty acid composition of tissues is related to body mass of mammals. *J. Membr. Biol.* **148**, 27-39. doi:10.1007/BF00234153

Dawson, W. R. and Olson, J. M. (2003). Thermogenic capacity and enzymatic activitues in the winter-acclimatized dark-eyed junco (Junco hyemalis). *J. Therm. Biol.* **28**, 497-508. doi:10.1016/S0306-4565(03)00050-0

Divakaruni, A. S. and Brand, M. D. (2011). The regulation and physiology of mitochondrial proton leak. *Physiology* **26**, 192-205. doi:10.1152/physiol.00046.2010

Duchamp, C., Marmonnier, F., Denjean, F., Lachuer, J., Eldershaw, T. PD., Rouanet, J. L., Morales, A., Meister, R., Benistant, C., Roussel, D., et al., (1999). Regulatory, cellular and molecular aspects of avian muscle nonshivering thermogenesis. *Ornis Fenn.* **76**, 151-165.

Else, P. L., Brand, M. D., Turner, N. and Hulbert, A. J. (2004). Respiration rate of hepatocytes varies with body mass in birds. *J. Exp. Biol.* **207**, 2305-2311. doi:10.1242/jeb.01017

Hill, R. W., Wyse, G. A. and Anderson, M. (2004). *Animal Physiology*. Sunderland, Massachusetts, USA: Sinauer Associates, Inc. publishers.

Hohtola, E. (2004). Shivering thermogenesis in birds and mammals. In *Life in the Cold: Evolution, Mechanisms, Adaptation, and Application. Twelfth International Hibernation Symposium* (ed. B. M. Barnes and H. V. Carey), pp. 241-252. Institute of Arctic Biology, University of Alaska Fairbanks.

Hohtola, E., Henderson, R. P. and Rashotte, M. E. (1998). Shivering thermogenesis in the pigeon: the effects of activity, diurnal factors, and feeding state. *Am. J. Physiol.* **275**, 1553-1562.

Hulbert, A. J. and Else, P. L. (1999). Membranes as possible pacemakers of metabolism. *J. Theor. Biol.* **199**, 257-274. doi:10.1006/jtbi.1999.0955

Hulbert, A. J., Faulks, S., Buttemer, W. A. and Else, P. L. (2002). Acyl composition of muscle membranes varies with body size in birds. *J. Exp. Biol.* **205**, 3561-3569. doi:10.1242/jeb.205.22.3561

Johnston, I., Calvo, J., Guderley, H., Fernandez, D. and Palmer, L. (1998). Latitudinal variation in the abundance and oxidative capacities of muscle mitochondria in perciform fishes. *J. Exp. Biol.* **201**, 1-12. doi:10.1242/jeb.201.1.1

Korzeniewski, B. (2015). "Idealized" state 4 and state 3 in mitochondria vs. rest and work in skeletal muscle. *PLoS One* **10**, 1-18.

Krijgsveld, K. L., Ricklefs, R. E. and Visser, G. H. (2012). Daily energy expenditure in precocial shorebird chicks: Smaller species perform at higher levels. *J. Ornithol.* **153**, 1203-1214. doi:10.1007/s10336-012-0851-1

Kuznetsov, A. V., Strobl, D., Ruttmann, E., Königsrainer, A., Margreiter, R. and Gnaiger, E. (2002). Evaluation of mitochondrial respiratory function in small biopsies of liver. *Anal. Biochem.* **305**, 186-194. doi:10.1006/abio.2002.5658

Kuznetsov, A. V., Veksler, V., Gellerich, F. N., Saks, V., Margreiter, R. and Kunz, W. S. (2008). Analysis of mitochondrial function in situ in permeabilized muscle fibers, tissues and cells. *Nat. Protoc.* **3**, 965-76. doi:10.1038/nprot.2008.61

Larsen, S., Nielsen, J., Hansen, C. N., Nielsen, L. B., Wibrand, F., Stride, N., Schroder, H. D., Boushel, R., Helge, J. W., Dela, F. et al. (2012). Biomarkers of mitochondrial content in skeletal muscle of healthy young human subjects. *J. Physiol.* **590**, 3349-3360. doi:10.1113/jphysiol.2012.230185

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**, 430-436. doi:10.1016/j.jtherbio.2011.07.011

Lowell, B. B. and Spiegelman, B. M. (2000). Towards a molecular understanding of adaptive thermogenesis. *Nature* **404**, 652-660. doi:10.1038/35007527

Marjoniemi, K. and Hohtola, E. (1999). Shivering thermogenesis in leg and breast muscles of galliform chicks and nestlings of the domestic pigeon. *Physiol. Biochem. Zool.* **72**, 484-492. doi:10.1086/316676

Mathers, K. E. and Staples, J. F. (2015). Saponin-permeabilization is not a viable alternative to isolated mitochondria for assessing oxidative metabolism in hibernation. *Biol. Open,* **4**, 858-864. doi:10.1242/bio.011544

Mathieu-Costello, O., Agey, P. J., Quintana, E. S., Rousey, K., Wu, L. and Bernstein, M. H. (1998). Fiber capillarization and ultrastructure of pigeon pectoralis muscle after cold acclimation. *J. Exp. Biol.* **201**, 3211-3220. doi:10.1242/jeb.201.23.3211

McKechnie, A. E. (2008). Phenotypic flexibility in basal metabolic rate and the changing view of avian physiological diversity: a review. *J. Comp. Physiol. B.* **178**, 235-247. doi:10.1007/s00360-007-0218-8

Mckechnie, A. E., Noakes, M. J. and Smit, B. (2015). Global patterns of seasonal acclimatization in avian resting metabolic rates (review). *J. Ornithol.* **156**, 367-376. doi:10.1007/s10336-015-1186-5

McKechnie, A. E. and Swanson, D. L. (2010). Sources and significance of variation in basal, summit and maximal metabolic rates in birds. *Curr. Zool.* **56**, 1-31. doi:10.1093/czoolo/56.6.741

Milbergue, M. S., Blier, P. U. and Vézina, F. (2018). Large muscles are beneficial but not required for improving thermogenic capacity in small birds. *Sci. Rep.* **8**, 14009.

Mozo, J., Emre, Y., Bouillaud, F., Ricquier, D. and Criscuolo, F. (2005). Thermoregulation: What Role for UCPs in Mammals and Birds? *Biosci. Rep.* **25**, 227-249. doi:10.1007/s10540-005-2887-4

Munro, D., Pichaud, N., Paquin, F., Kemeid, V. and Blier, P. U. (2013). Low hydrogen peroxide production in mitochondria of the long-lived Arctica islandica: underlying mechanisms for slow aging. *Aging Cell* **12**, 584-592. doi:10.1111/acel.12082

Murrell, P. (2005). *R Graphics*. Boca Raton, Florida: Chapman & Hall/CRC.

Nicholls, D. G. and Ferguson, S. J. (1992). *Bioenergetics 2*. San Diego: Academic press.

Nielsen, J., Gejl1, K. D., Hey-Mogensen, M., Holmberg, H.-C., Suetta, C., Krustrup, P., Elemans, C. P. H. and Ørtenblad, N. (2017). Plasticity in mitochondrial cristae density allows metabolic capacity modulation in human skeletal muscle. *J.Physiol.* **584.9**, 2839-2847. doi:10.1113/JP273040

Nisoli, E., Clementi, E., Paolucci, P., Cozzi, V., Tonello, C., Sciorati, C., Bracale, R., Valerio, A., Francolini, M., Moncada, S. et al. (2003). Mitochondrial biogenesis in mammals: the role of endogenous nitric oxide. *Science* **299**, 896-899. doi:10.1126/science.1079368

O'Connor, T. P. (1995). Metabolic characteristics and body composition in house finches: effects of seasonal acclimatization. *J. Comp. Physiol. B* **165**, 298-305. doi:10.1007/BF00367313

Peña-Villalobos, I., Nuñez-Villegas, M., Bozinovic, F. and Sabat, P. (2014). Metabolic enzymes in seasonally acclimatized and cold acclimated rufous-collared sparrow inhabiting a Chilean Mediterranean environment. *Curr. Zool.* **60**, 338-350. doi:10.1093/czoolo/60.3.338

Pesta, D. and Gnaiger, E. (2011). Preparation of permeabilized muscle fibers for diagnosis of mitochondrial respiratory function. *Mitochondrial Physiol. Netw.* **14**, 1-4.

Petit, M., Lewden, A. and Vezina, 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**, 539-549. doi:10.1086/676669

Picard, M., Ritchie, D., Wright, K. J., Romestaing, C., Thomas, M. M., Rowan, S. L., Taivassalo, T. and Hepple, R. T. (2010). Mitochondrial functional impairment with aging is exaggerated in isolated mitochondria compared to permeabilized myofibers. *Aging Cell* **9**, 1032-1046. doi:10.1111/j.1474-9726.2010.00628.x

Picard, M., Ritchie, D., Thomas, M. M., Wright, K. J. and Hepple, R. T. (2011). Alterations in intrinsic mitochondrial function with aging are fiber type-specific and do not explain differential atrophy between muscles. *Aging Cell* **10**, 1047-1055. doi:10.1111/j.1474-9726.2011.00745.x

Picard, M., Hepple, R. T. and Burelle, Y. (2012). Mitochondrial functional specialization in glycolytic and oxidative muscle fibers: tailoring the organelle for optimal function. *Am. J. Physiol. Cell Physiol.* **302**, C629-C641. doi:10.1152/ajpcell.00368.2011

Porter, R. K., Hulbert, A. J. and Brand, M. D. (1996). Allometry of mitochondrial proton leak: influence of membrane surface area and fatty acid composition. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **271**, 1550-1560. doi:10.1152/ajpregu.1996.271.6.R1550

Rabøl, R., Larsen, S., HRabøljberg, P. M. V., Almdal, T., Boushel, R., Haugaard, S. B., Andersen, J. L., Madsbad, S. and Dela, F. (2010). Regional anatomic differences in skeletal muscle mitochondrial respiration in Type 2 diabetes and obesity. *J. Clin. Endocrinol. Metab*. **95**, 857-863. doi:10.1210/jc.2009-1844

Rising, J. D. and Hudson, J. W. (1974). Seasonal variation in the metabolism and thyroid activity of the black-capped chickadee (Parus atricapillus). *Condor* **76**, 198-203. doi:10.2307/1366730

Rolfe, D. F. S. and Brand, M. D. (1997a). The physiological significance of mitochondrial proton leak in animal cells and tissues. *Biosci. Rep.* **17**, 9-16. doi:10.1023/A:1027327015957

Rolfe, D. F. S. and Brown, G. C. (1997b). Cellular energy utilization and molecular origin of standard metabolic rate in mammals. *Physiol. Rev.* **77**, 731-758. doi:10.1152/physrev.1997.77.3.731

Rolfe, D. F., Newman, J. M., Buckingham, J. A., Clark, M. G. and Brand, M. D. (1999). Contribution of mitochondrial proton leak to respiration rate in working skeletal muscle and liver and to SMR. *Am. J. Physiol.* **276**, 692-699. doi:10.1152/ajpcell.1999.276.3.C692

Roussel, D., Rouanet, J. L., Duchamp, C. and Barre, H. (1998). Effects of cold acclimation and palmitate on energy coupling in duckling skeletal muscle mitochondria. *FEBS Lett.* **439**, 258-262. doi:10.1016/S0014-5793(98)01382-9

Roussel, D., Salin, K., Dumet, A., Romestaing, C., Rey, B. and Voituron, Y. (2015). Oxidative phosphorylation efficiency, proton conductance and reactive oxygen species production of liver mitochondria correlates with body mass in frogs. *J. Exp. Biol.* **218**, 3222-3228. doi:10.1242/jeb.126086

Saarela, S., Klapper, B. and Heldmaier, G. (1995). Daily rhythm of oxygen consumption and thermoregulatory responses in some European winter- or summer-acclimatized finches at different ambient temperatures. *J. Comp. Physiol. B* **165**, 366-376. doi:10.1007/BF00387307

Salin, K., Auer, S. K., Rudolf, A. M., Anderson, G. J., Cairns, A. G., Mullen, W., Hartley, R. C., Selman, C. and Metcalfe, N. B. (2015). Individuals with higher

metabolic rates have lower levels of reactive oxygen species *in vivo*. *Biol. Lett.* **11**, 20150538. doi:10.1098/rsbl.2015.0538

Salin, K., Auer, S. K., Rudolf, A. M., Anderson, G. J., Selman, C. and Metcalfe, N. B. (2016). Variation in metabolic rate among individuals is related to tissuespecific differences in mitochondrial leak respiration. *Physiol. Biochem. Zool.* **89**, 511-523. doi:10.1086/688769

Scott, G. R., Richards, J. G. and Milsom, W. K. (2009). Control of respiration in flight muscle from the high-altitude bar-headed goose and low-altitude birds. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **297**, 1066-1074. doi:10.1152/ajpregu.00241.2009

Silva, J. E. (2003). The thermogenic effect of thyroid hormone and its clinical implications (review). *Physiol. Med.* **139**, 205-213.

St-Pierre, J., Charest, P.-M. and Guderley, H. (1998). Relative contribution of quantitative and qualitative changes in mitochondria to metabolic compensation during seasonal acclimatisation of rainbow trout Oncorhynchus Mykiss. *J. Exp. Biol.* **201**, 2961-2970. doi:10.1242/jeb.201.21.2961

Stager, M., Swanson, D. L. and Cheviron, Z. A. (2015). Regulatory mechanisms of metabolic flexibility in the dark-eyed junco (Junco hyemalis). *J. Exp. Biol.* **218**, 767-777. doi:10.1242/jeb.113472

Swanson, D. L. (2010). Seasonal metabolic variation in birds: functionnal and mechanistic correlates. *Current Ornithol* **17**, 75-129. doi:10.1007/978-1-4419-6421-2_3

Swanson, D. L. and Liknes, E. T. (2006). A comparative analysis of thermogenic capacity and cold tolerance in small birds. *J. Exp. Biol.* **209**, 466-474. doi:10.1242/jeb.02024

Swanson, D. L. and Vézina, F. (2015). Environmental, ecological and mechanistic drivers of avian seasonal metabolic flexibility in response to cold winters. *J. Ornithol.* **156**, 377-388. doi:10.1007/s10336-015-1192-7

Swanson, D. L., Zhang, Y. and King, M. O. (2013). Individual variation in thermogenic capacity is correlated with flight muscle size but not cellular metabolic capacity in American goldfinches (Spinus tristis). *Physiol. Biochem. Zool.* **86**, 421-431. doi:10.1086/671447

Swanson, D., Zhang, Y. and King, M. (2014a). Mechanistic drivers of flexibility in summit metabolic rates of small birds. *PLoS One* **9**, e101577. doi:10.1371/journal.pone.0101577

Swanson, D. L., Zhang, Y., Liu, J.-S., Merkord, C. L. and King, M. O. (2014b). Relative roles of temperature and photoperiod as drivers of metabolic flexibility in dark-eyed juncos. *J. Exp. Biol.* **217**, 866-875. doi:10.1242/jeb.096677

Swanson, D. L., King, M. O., Culver, W. and Zhang, Y. (2017). Within-winter flexibility in muscle masses, myostatin, and cellular aerobic metabolic intensity in passerine birds. *Physiol. Biochem. Zool.* **90**, 210-222. doi:10.1086/688956

Teulier, L., Rouanet, J.-L., Letexier, D., Romestaing, C., Belouze, M., Rey, B., Duchamp, C. and Roussel, D. (2010). Cold-acclimation-induced non-shivering thermogenesis in birds is associated with upregulation of avian UCP but not with innate uncoupling or altered ATP efficiency. *J. Exp. Biol.* **213**, 2476-2482. doi:10.1242/jeb.043489

Thibault, M., Blier, P. U. and Guderley H. (1997). Seasonal variation of muscle metabolic organization in rainbow trout (Oncorhynchus mykiss). *Fish Physiol. Biochem.* **16**, 139-155.

Vézina, F. and Williams, T. D. (2005). Interaction between organ mass and citrate synthase activity as an indicator of tissue maximal oxidative capacity in breeding European Starlings : implications for metabolic rate and organ mass relationships. *Funct. Ecol.* **19**, 119-128. doi:10.1111/j.0269-8463.2005.00942.x

Vézina, F., Dekinga, A. and Piersma, T. (2011). Shorebirds' seasonal adjustments in thermogenic capacity are reflected by changes in body mass: How preprogrammed and instantaneous acclimation work together. *Integr. Comp. Biol.* **51**, 394-408. doi:10.1093/icb/icr044

Vézina, F., Gerson, A. R., Guglielmo, C. G. and Piersma, T. (2017). The performing animal: causes and consequences of body remodeling and metabolic adjustments in red knots facing contrasting thermal environments. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **313**, 120-131. doi:10.1152/ajpregu.00453.2016

Walker, J. M. (1994). The bicinchoninic acid (BCA) assay for protein quantitation. *Methods Mol Biol.* **32**, 5-8. doi:10.1385/0-89603-268-X:5

West, G. B., Woodruff, W. H. and Brown, J. H. (2002). Allometric scaling of metabolic rate from molecules and mitochondria to cells and mammals. *Proc. Natl. Acad. Sci. USA* **99**, 2473-2478. doi:10.1073/pnas.012579799

Williams, J. B. and Tieleman, B. I. (2000). Flexibility in basal metabolic rate and evaporative water loss among hoopoe larks exposed to different environmental temperatures. *J. Exp. Biol.* **203**, 3153-3159. doi:10.1242/jeb.203.20.3153

Zhang, Y., Eyster, K. and Swanson, D. L. (2017). Context-dependent regulation of pectoralis myostatin and lipid transporters by temperature and photoperiod in darkeyed juncos. *Curr. Zool.* **64**, 23-31. doi:10.1093/cz/zox020

Zheng, W.-H., Li, M., Liu, J.-S. and Shao, S.-L. (2008). Seasonal acclimatization of metabolism in Eurasian tree sparrows (Passer montanus). *Comp. Biochem. Physiol. A. Mol. Integr. Physiol.* **151**, 519-525. doi:10.1016/j.cbpa.2008.07.009

Zheng, W.-H., Lin, L., Liu, J.-S., Pan, H., Cao, M.-T. and Hu, Y.-L. (2013). Physiological and biochemical thermoregulatory responses of Chinese bulbuls Pycnonotus sinensis to warm temperature: Phenotypic flexibility in a small passerine. *J. Therm. Biol.* **38**, 240-246. doi:10.1016/j.jtherbio.2013.03.003

Zheng, W.-H., Liu, J.-S. and Swanson, D. L. (2014). Seasonal phenotypic flexibility of body mass, organ masses, and tissue oxidative capacity and their relationship to resting metabolic rate in Chinese bulbuls. *Physiol. Biochem. Zool.* **87**, 432-444. doi:10.1086/675439

Zhou, L.-M., Xia, S.-S., Chen, Q., Wang, R.-M., Zheng, W.-H. and Liu, J.-S. (2016). Phenotypic flexibility of thermogenesis in the hwamei (Garrulax canorus): responses to cold acclimation. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **310**, 330-336. doi:10.1152/ajpregu.00259.2015

Table S1. Effect of treatments and body mass on mitochondrial LEAK, $OXPHOS_{CI}$ and $OXPHOS_{CI+CII}$ in the pectoralis muscle. These values were obtained using ANCOVA models that included the effect of thermal treatment and the effect of body mass as a covariate. Models also included the interaction treatment*body mass (all n.s, not presented here).

	Cold	**Thermoneutral**	**Treatment**		**Body mass**	
	Mean ± s.e.m (pmol O_2.sec^{-1})		F (df)	P	F (df)	P
LEAK /mg tissue	12.0±1.1	12.2±1.0	0.0 (1,31)	0.9	0.18 (1,29)	0.7
/ CS	10.6±1.2	12.3±1.1	1.1 (1,28)	0.3	0.0 (1,26)	1.0
/ CCO	209.7±30.6	247.0±28.7	0.79 (1,30)	0.4	0.27 (1,28)	0.6
$OXPHOS_{CI}$ /mg tissue	23.7±4.7	24.1±4.2	0.0 (1,27)	0.95	0.25 (1,25)	0.6
/CS	20.9±4.5	24.1±4.2	0.26 (1,24)	0.6	0.12 (1,22)	0.7
/CCO	442.8±134.1	505.0±124.8	0.11 (1,26)	0.7	0.12 (1,24)	0.7
$OXPHOS_{CI+CII}$ /mg tissue	56.6±7.7	49.5±7.1	0.46 (1,31)	0.5	0.5 (1,29)	0.5
/CS	44.2±6.9	50.7±6.5	0.46 (1,28)	0.5	0.57 (1,26)	0.5
/CCO	994.1±191.3	1040.7±179.7	0.03 (1,30)	0.9	0.31 (1,28)	0.6
RCR	2.3±0.34	2.1±0.33	0.1 (1,25)	0.7	0.18 (1,23)	0.7

Journal of Experimental Biology • Supplementary information