MONASH University

Mitochondria and Metabolism as Drivers of Climate Adaptation in Wild Populations of Eastern Yellow Robin

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MSc Biodiversity and Systematics

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A thesis submitted for the degree of *Doctor of Philosophy*

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To my brother,

Who, by daring me, insinuated the idea of doing a PhD into my subconsciousness

before I had even entered university.

Copyright notice

Abstract

Although species constantly change to suit their climatic envelopes, anthropogenically accelerated rates of climate change are challenging their adaptive pace. A better understanding of the mechanisms of climate adaptation is needed to inform policy-makers and improve conservation outcomes. In different climates, optimal use of energy differs as well as the overall energetic cost of living. Thus, the adaptation of energy-metabolising pathways is an important avenue to overcome climatic challenges.

This thesis investigated the role of mitochondria in the climate adaptation of these metabolic pathways. Mitochondria harbour the protein machinery that operates the oxidative phosphorylation, and the mitochondrial genome encodes a modest proportion of the protein involved in this pathway. Oxidative phosphorylation provides the vast majority of cellular useable energy through the oxidation of nutrient metabolites. Consequently, it is generally hypothesised that mitochondrial genetic variation affects rates and efficiencies of mitochondrial energy metabolism, and thereby that of the whole organism.

By utilising the diverged and strongly climate-correlated mitochondrial lineages of a bird, the Eastern Yellow Robin (*Eopsaltria australis*), I tested the association of mitochondrial genetic variation with mitochondrial function and metabolic characteristics in free-living individuals. Using high-resolution respirometry of blood to measure mitochondrial function did not reveal an association with mitochondrial lineage, once the association between lineage distribution and climate was accounted for. However, it showed that Eastern Yellow robins increase mitochondrial metabolic rate and decrease efficiency in more mesic conditions (both long- and short-term).

In contrast, mitochondrial lineage explained seasonal differences in the adjustment of whole-organism metabolic rates. Individuals of the lineage typically experiencing cooler climates had a higher resting metabolism than the individuals of the lineage typically found in hotter conditions. These metabolic rates were also predicted by ambient temperature experienced in the weeks before measurement, but not humidity. However, increasing levels of humidity did show lower evaporative water loss in simultaneous investigations of the whole-organism.

Finally, I provide evidence that mitochondrial metabolism affects whole-organism metabolic rates via cellular mechanisms. The metabolic rate of intact blood cells did show a positive effect on whole-organism metabolism, while the activities of enzymes of oxidative phosphorylation did not.

In conclusion, I found support for the involvement of mitochondria in shaping metabolic responses potentially relevant to climate adaptation; (1) mitochondrial function affected basal metabolism of the whole organism, and (2) mitochondrial genetic differences likely conveyed adjustment of basal metabolic rate between seasons. I furthermore, show support for the importance of humidity in metabolic responses to climate. Mitochondrial genetic variation and humidity have rarely been highlighted as potential drivers of metabolic adaptation (and thus climate adaptation) but should be more strongly considered in ecophysiology and in its application to conserve species more effectively under climate change.

Publications during Enrolment

Gan, HM, Falk, S, Morales, HE, Austin, CM, Sunnucks, P, Pavlova, A. (Submitted) Genomic evidence of neo-sex chromosomes in the Eastern Yellow Robin. *GigaScience*.

Declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis includes one submitted publication. The core theme of the thesis is the role of mitochondria in climate adaptation. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the School of Biological Sciences under the supervision of *Prof Paul Sunnucks, Dr Alexandra Pavlova,* and *Prof Craig White*.

The inclusion of co-authors reflects the fact that the work came from active collaborations between researchers and acknowledges input into team-based research.

In the case of Chapters 2 - 4 my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author names Nature and % of Co-author's contribution	Co-author Monash student?
2	Acclimation, Climate and Mitolineage as Predictors of Mitochondrial Function in a Wild Bird	Submitted (In revision)	85% Design, general field work, data collection, data analysis, and manuscript preparation	Antoine Stier: Methodological design, manuscript revision (5%) Craig White: Manuscript revision (2.5%) Alexandra Pavlova: Design, manuscript revision (2.5%) Paul Sunnucks: Design, manuscript revision (5%)	No
3	Linking mitochondrial genetic variation to metabolic rate and evaporative water loss in a passerine	In prep.	85% Design, general field work, data collection, data analysis, and manuscript preparation	Craig White: Design, manuscript revision (5%) Alexandra Pavlova: Design, manuscript revision (5%) Paul Sunnucks: Design, manuscript revision (5%)	No
4	Blood Cell Respiration	In prep.	90%	Antoine Stier: Manuscript revision (2.5%)	

	predicts Basal Metabolic Rate		Design, general field work, data collection, data analysis, and manuscript preparation	Craig White: Manuscript revision (2.5%) Alexandra Pavlova: Manuscript revision (2.5%) Paul Sunnucks: Manuscript revision (2.5%)	No

I have renumbered sections of submitted or published papers in order to generate a consistent presentation within the thesis.

Student name: Stephanie Falk

Student signature: **Date:** 26/06/2019

I hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author, I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor name: Prof Paul Sunnucks

Main Supervisor signature: **Date:** 20/06/2019

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Thank you, panel. Your input has certainly shaped the project in the best possible way. Your kind words were a great support and helped me to move on from the lower points of this journey.

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CHAPTER 1

General Introduction

Why and how to study mitochondria in the context of climate adaptation

Stephanie Falk

Studying the role of mitochondria in climate adaptation

Understanding adaptation under climate change

Climate adaptation - the changing of ecosystems, species and populations to avoid negative impacts and foster benefits under different climatic conditions - assists species to persist long-term. Although species will constantly change to suit shifting climatic envelopes, anthropogenically accelerated rates of climate change may exceed the rates at which species can adapt to them (Urban 2015). Species are faced with manifold challenges, not only global warming, but also increasingly arid and extreme climate and weather, as well as asynchronization of crucial life history events and changes in resource availability (Tarwater and Beissinger 2013; Plard *et al.* 2014; Scheffers *et al.* 2016; Senner *et al.* 2018). For many species, this evolutionary arms-race has been predicted to end in their defeat (Moritz *et al.* 2008; Lee *et al.* 2015; Tilman *et al.* 2017).

There are many benefits to understanding the process of climate adaptation. One is the ability to help populations cope under anthropogenic climate change. As we learn more about which attributes of an individual are impacted by different climate characteristics, we are enabled to develop more effective approaches to conservation management (Prober *et al.* 2012; Broadhurst *et al.* 2015). Furthermore, understanding climate adaptation also helps to understand speciation. When different climatic pressures are imposed on different populations of a species, selection can drive divergence (Keller and Seehausen 2012). However, whether or not divergent climate adaptation can cause sufficient barriers to gene-flow to restrict admixture of diverged populations on secondary contact has not been shown coherently (Qvarnstrom *et al.* 2016). Understanding the mechanisms of climate adaptation will be crucial for answering this question.

The role of mitochondria in climate adaptation

Our understanding of adaptation changed with the discovery of the nuclear genome, and may receive yet another twist as we learn more about the impact of the mitochondrial genome (mitogenome). The mitogenome was thought to evolve neutrally, but this this view has been increasingly challenged over recent decades (Rand *et al.* 1994; Dowling *et al.* 2008; Horan *et al.* 2013). We are now seeing evidence that links mitochondrial genotype to phenotypic differences (Blier *et al.* 2012; Ballard *et al.* 2014; Vijayraghavan *et al.* 2019). Metabolism is a common trait, or rather a suite of traits, studied in the context of mitochondrial genetic

variation (Albertin *et al.* 2013; Toews *et al.* 2014b; Jin *et al.* 2018). All products of protein-coding genes of the mitogenome are part of the oxidative phosphorylation system. This biochemical pathway converts the energy from ingested nutrients to cellular usable ATP (adenosine triphosphate), and provides 90 percent of the energy demand in most organisms (Rolfe and Brown 1997). Thus, oxidative phosphorylation underpins all aspects of life, because to live is to expend energy. In a narrower sense, oxidative phosphorylation is thought to be the basis of an individual's metabolic rate (Tieleman *et al.* 2009; Blier *et al.* 2014) - the rate of energy expenditure of the entire organism for a given unit of time.

Under the assumption of limited resources, an individual's fitness is expected to be affected by how much and how efficiently it uses energy to sustain itself and reproduce (Lillywhite 2006; White *et al.* 2007a; Burton *et al.* 2011). However, the optimal metabolic rate of an individual at a given point in time depends on its life stage and environment (Pettersen *et al.* 2018b). In relation to climate, most expectations and observations of optimal metabolism are focused on variation in temperature and latitude (Tieleman *et al.* 2003b; McKechnie 2008). Also, most studies focus on a sole measure of metabolism: basal metabolic rate (BMR) in endotherms, or its equivalent in ectotherms, standard metabolic rate. Both are metabolic rates under strictly defined conditions that give estimates for the lowest sustainable metabolic rate - often thought to represent the minimum cost of living (Willmer 2000).

Insights into the potential adaptive significance of metabolic rate might be obtained by considering their variation in geographic and environmental space. Generally, species that live in warmer environments have lower BMRs than those that live in cooler environments (Tieleman *et al.* 2003b; McKechnie 2008). This suggests that climate adaptation of BMR enables thermal homeostasis in individuals across a wide range of ambient temperatures. This is most commonly explained as an increase of metabolic heat production in cold environments, and the minimization of metabolic heat production in hot environments (Humphries *et al.* 2005; Naya *et al.* 2018).

Investigating mitochondria-mediated climate adaptation in the wild

In wild populations, mitochondrial genetic variation has rarely been linked to variation in metabolic rate (Norin and Metcalfe 2019). Studying climate adaptation under natural rather than controlled conditions enables simultaneous assessment of many climatic variables and their interactions. Studies in the wild cannot resolve effects of environmental variables that are confounded with climate; however, when the interest is in understanding climate adaptation in

the wild, these associations of environmental variables are important components of natural systems. The most all-encompassing approach to assess climate adaptation is to compare the fitness of different genotypes (say A and B) side-by-side in their different habitats, one where A is 'local' and B is 'immigrant', one where A is 'immigrant' but B is 'local'. This can be achieved by reciprocal translocations of individuals (Kawecki and Ebert 2004; McFarlane *et al.* 2018). Even in species for which translocations are feasible, such movements are often viewed as a risk to local adaptation in the recipient population, and their implementation can be challenging for reasons relating to regulations and ethical considerations (Weeks *et al.* 2011). Alternatively, putative climate-adapted phenotypes can be studied in shared environments that are not 'home' for either genotype. However, this may not be available to many natural systems, and most importantly, the fitness of genotypes in question depend on the habitat quality of shared environment relative to their 'home' environment (Kawecki and Ebert 2004). In conclusion, when studying mitochondria-mediated climate adaptation in a wild setting, a suitable model system is crucial, while the most applicable design and methods used for these studies depend on the ecosystem in question as well as feasibility.

The Eastern Yellow Robin as a wildlife model for mitochondria-mediated climate adaptation

The Eastern Yellow Robin is an endemic eastern Australian songbird. It combines several attributes useful for the study of climate adaptation in the light of mitochondrial genotypic variation. In the southern parts of its distribution, the Eastern Yellow Robin harbours two substantially diverged mitochondrial lineages (mitolineages, mito-A and mito-B). They inhabit largely separate geographic ranges that co-vary strongly with climate (Figure 1). However, individuals of populations bearing the different mitolineages co-exist in narrow hybrid zones (Pavlova *et al.* 2013). The 6.8% nucleotide difference between the whole mitogenomes of the mitolineages includes five amino acid replacements in the oxidative phosphorylation Complex I that are fixed between the lineages and inferred to be under positive selection (Morales *et al.* 2015; Lamb *et al.* 2018). These are predicted to significantly change protein physio-chemical properties, which may impact how oxidative phosphorylation functions (Morales *et al.* 2015; Lamb *et al.* 2018). The mitolineages and their geographical distributions suggest that they were shaped by mitochondria-mediated climate adaptation (Morales *et al.* 2015; Sunnucks *et al.* 2017).

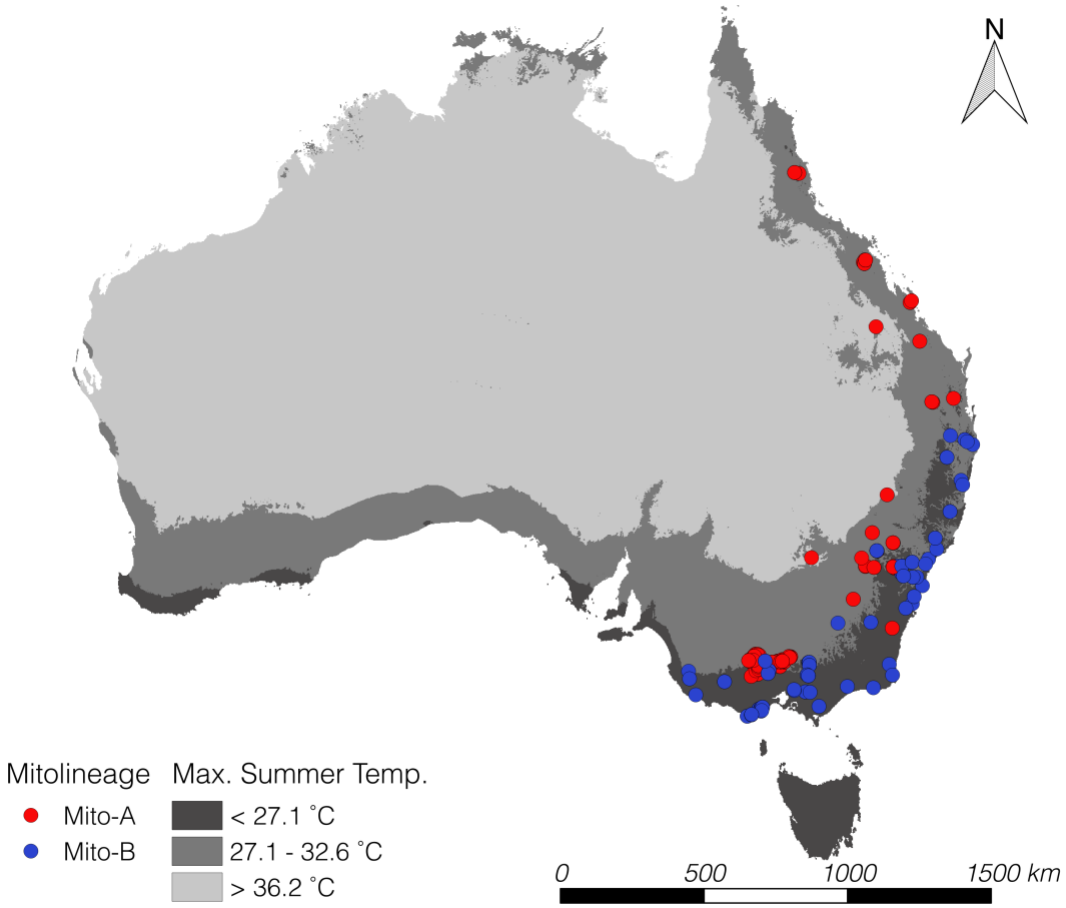

Figure 1: Distribution of Eastern Yellow robin mitolineages across the range of the species. Mito-A individuals are commonly found in the hotter, more arid, and more climatically variable inland. In contrast, mito-B individuals are commonly found towards the coast, in more mesic and stable climatic environments.

Thesis scope and outline

To test whether mitochondrial genetic variation in the Eastern Yellow Robin gives rise to metabolic phenotypes potentially conveying climate adaptation, we utilised the framework in Sunnucks *et al.* (2017), which formulates expectations of responses to climatic pressures on various levels of biological organization. In particular, we focused on linking phenotypic variation in metabolic traits to the two mitolineages of Eastern Yellow Robin.

The framework adopted here focussed on mitochondria as drivers for climate adaptation (Figure 2). In the Eastern Yellow Robin, we expected that the amino acid differences between the two mitolineages would lead to structural changes in the oxidative phosphorylation complex I. These changes would in particular affect how efficiently ADP was phosphorylated, and how much metabolic heat was released in the process. We hypothesized that these differences in energy metabolism at least partially determine the metabolic rate of the individual. We then assumed that individual whole-organism metabolism is a trait under selection for climate adaptation.

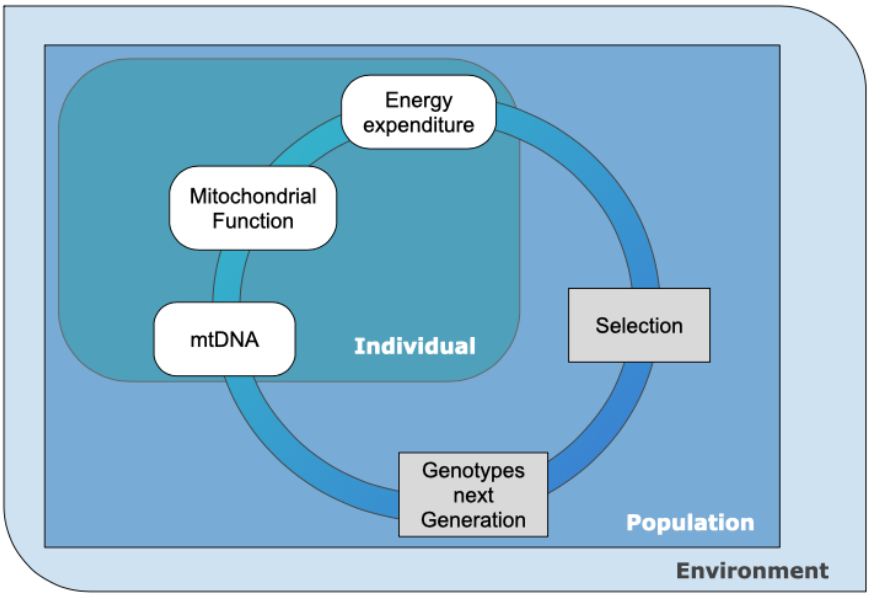

Figure 2: Investigated framework of mitochondria-mediated climate adaptation (based on Sunnucks et al. 2017). We expected that within the individual, mitochondrial genetic variation (mtDNA) causes differences in mitochondrial function, likely via altered proteins of the oxidative phosphorylation pathway. This variation would then carry through to the level of the entire organism, where mitochondrial function affects metabolic rate (energy expenditure). We expected that metabolic rate of the individual is a trait on which selection can act. At the population level, individuals of different metabolic rate would differentially contribute to the genotypes of the next generation. This would then close the circle of mitochondria-mediated climate adaptation, by determining the mitochondrial genetic variation found in individuals of the new generation.

To test the outlined chain of function we studied four populations. These were located in the southern part of the species' distribution, where both mitolineages are found in close proximity to each other. Two locations each harboured only one of the two mitolineages (referred to as Pure-Hot and Pure-Cold). In the other two locations, both mitolineages co-inhabited and inter-bred.

These shared locations had an intermediate climate of the two pure sites, but differed slightly from each other. One shared site was hotter and more variable in climate than the other and had a higher proportion of mito-A (hot type) individuals (Shared-Hot site). The other shared site comprised a higher proportion of mito-B (cold type) individuals (Shared-Cold site).

These shared locations were useful to compare the metabolic phenotypes of individuals of different mitolineages within the same environment, eliminating phenotypic plasticity caused by environmental differences, thus isolating putative mitolineage effects. The inclusion of both shared sites is relevant, because each site has a climate profile closer to one of the pure sites (e.g. Shared-Hot closer to Pure-Hot). This allowed comparisons of the mitolineages between environments to which they are putatively adapted, and environments to which the other mitolineage is putatively adapted, e.g. mito-A (hot type) and mito-B (cold type) individuals in a relatively hot site (Shared-Cold) that should be more suitable for mito-A.

Assuming this model of chain of function of mitochondria-mediated climate adaptation for the Eastern Yellow Robin, we derive the following expectations:

A. Mitochondrial function of an Eastern Yellow robin is predicted by its mitolineage. The individuals in the Pure-Hot sites will have more efficient oxidative phosphorylation systems, releasing less heat, compared to individuals in the Pure-Cold site. Individuals of the different mitolineages should retain these characteristics in the shared sites, unless environmentally induced metabolic plasticity, local adaptation and genetic admixture are sufficient to cause them to converge on the same phenotype at shared sites irrespective of their mitolineage.
B. The BMR of an Eastern Yellow robin is predicted by its mitolineage. The individuals in the Pure-Hot sites should have significantly lower BMR than do those in the Pure-Cold site. For the reasons above, this pattern may be less pronounced in the shared sites.
C. The BMR of Eastern Yellow robins is predicted by variation in mitochondrial function. Individuals that have more efficient oxidative phosphorylation systems will also have lower BMR. This relationship may be less strong in the shared sites, as above.

These expectations formed the basis of the three main chapters (Chapters 2-4, Figure 3) of this thesis:

Chapter 2 focuses on whether mitochondrial function differs between the mitolineages. It also tests the relevance of a large number of climatic variables to mitochondrial function using novel bio-statistical tools. This is an important step, because mitolineages and climate are largely spatially confounded. Furthermore, variables of weather conditions just prior to the metabolic assessments of the individuals are included to account for phenotypic plasticity.

Chapter 3 investigates the drivers of whole-organism metabolic rates and evaporative water loss. It assesses environmental and genetic effects on BMR, and also the metabolic profile of Eastern Yellow robins over a range of environmentally relevant temperatures. Again, climate and weather are tested as potential drivers.

Chapter 4 connects the cellular processes (as in Chapter 2) to the entire organism (as in Chapter 3) by assessing correlations between different measures of mitochondrial function and BMR taken in the same individuals on the same occasions.

Chapter 5 integratively assesses the contributions made by this thesis to the understanding of climate adaptation, eco-physiology, and bioenergetics in the light of different climatic pressures. It highlights unexpected and novel findings, and uses these to lay out future directions for studies investigating climate adaptation considering mitochondrial genetic variation and mitochondrial function.

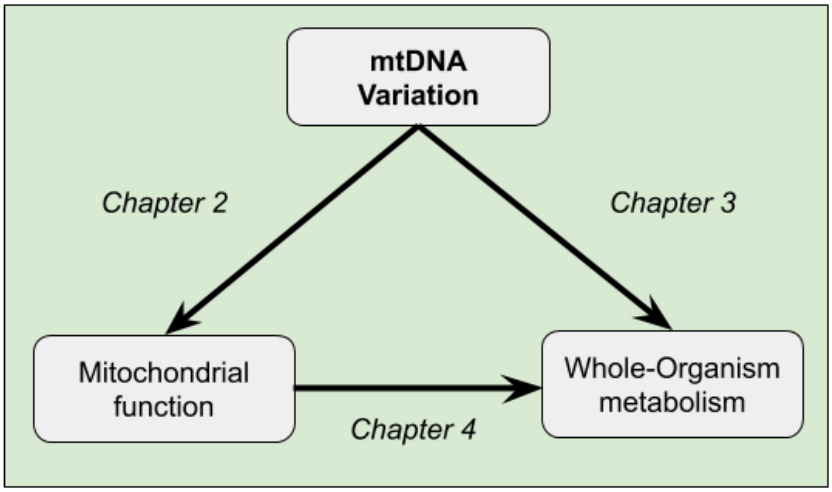

Figure 3: Graphical representation of the three data-rich and analytical chapters. Chapter 2 identified relevant features affecting mitochondrial function assessed by different measures of OXPHOS activity. The putative effects of mtDNA variation were contrasted to environmental and individual-specific factors. Chapter 3 identified likely contributors shaping whole-organism metabolic rate as well as evaporative water loss. Apart from mtDNA variation, environmental and individual specific factors were included as potential contributors. Chapter 4 assessed whether the relationships suggested as important for mitochondrial and whole-body metabolism by the previous two chapters form a coherent and plausible framework. It had a particular focus on linking variation in mitochondrial function to whole-organism metabolic phenotypes.

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CHAPTER 2

Acclimation, Climate and Mitolineage as Predictors of Mitochondrial Function in a Wild Bird

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Antoine Stier

Alexandra Pavlova

Craig R. White

Paul Sunnucks

Abstract

Mitochondria are expected to play a role in climate adaptation because they host and co-regulate oxidative phosphorylation, which is the main pathway of cellular energy conversion. How efficiently energy in form of ATP is synthesized in this process could convey adaptation to different climates, because inefficiency increases the relative amount of metabolic heat dissipated. In our study species, the Eastern Yellow Robin (EYR), two climate-associated mitochondrial lineages (mitolineages) harbour oxidative phosphorylation proteins that are likely to change ATP synthesis efficiency. Thus, the mitolineages might produce climate-adapted phenotypes. We assessed whether mitolineage predicts mitochondrial function, while controlling for acclimation. We measured mitochondrial function of blood cell mitochondria in wild populations using high-resolution respirometry. We did not detect a difference in metabolic function between the lineages after considering long- and short-term variation in climate. At locations where individuals of both mitolineage co-inhabit the same environment, EYR also interbreed. Thus, mito-nuclear incompatibilities in inter-lineage hybrids might negate the climate-adaptive benefit encoded in the mitochondrial DNA in these locations. Across all study sites, oxidative phosphorylation efficiency was strongest and positively associated with long-term climate variables related to environmental water turn-over rates in the coolest months. To separate the effect of mitolineage and climate, manipulative experiments are required.

Introduction

Linking genotype to phenotype is a fundamental approach to understanding adaptation. Mitochondrial DNA variation is increasingly being demonstrated to convey adaptive phenotypes (Rand *et al.* 1994; Ballard and Kreitman 1995; Dowling *et al.* 2008), based on the important roles of mitochondria and mitochondrially-encoded proteins in oxidative phosphorylation (Fangue *et al.* 2009; Ballard *et al.* 2014; Ben Slimen *et al.* 2018). Mitochondria host, co-encode, and co-regulate this core metabolic pathway that provides the vast majority of cellular energy in the form of ATP (Brand 1990).

A possible way in which genetic variation in oxidative phosphorylation could convey climate adaptation lies in the modulation of *coupling*: the efficiency with which the chemiosmotic proton gradient produced by the oxidation of electron donors drives ATP synthesis (Portner 2002; Ballard and Melvin 2010; Campbell-Staton *et al.* 2018). Higher coupling means more of the gradient is used for ATP synthesis and less is lost to the exothermic backflow of protons along the gradient (termed *proton leak*). The optimal balance between heat dissipation and ATP synthesis is thought to depend on local climatic pressures (Gershoni *et al.* 2009; Ballard and Melvin 2010). In addition to coupling (a ratio), the maximum rates of ATP synthesis and heat release are likely to determine an individual's optimal climatic environment (Portner 2002). This is particularly true for endotherms living in extreme conditions, where thermoregulation demands a large proportion of available energy. For example, in consistently colder climates an increase in the rate of proton leak and thus heat dissipation can be beneficial. In comparison, in more variable hotter climates, an increase in the rate of proton leak and/or decrease in efficiency (higher rate of proton relative to ATP production) might be maladaptive (Sunnucks *et al.* 2017).

Differences in metabolic activity have rarely been linked to mitochondrial genetic variation (Fangue *et al.* 2009; Ballard *et al.* 2014; Wolff *et al.* 2016), especially in wild organisms (Oellermann *et al.* 2012; Toews *et al.* 2014a; Stager *et al.* 2016). To be able to explore these associations in wild populations, a model system is needed that harbours mitochondrial genetic variation associated with climate in a relatively uniform nuclear background. The Eastern Yellow Robin (*Eopsaltria australis,* hereafter EYR), an endemic eastern Australian songbird, is a suitable system for testing the link between mitochondrial DNA variation and metabolic phenotype.

Eastern Yellow Robins harbor harbors two mitochondrial lineages (mitolineages, mito-A and mito-B) with 6.8% nucleotide divergence between their mitochondrial genomes. Despite nuclear gene flow, birds of different mitolineage have largely separate geographic ranges, but co-exist and hybridize in narrow contact zones (Pavlova *et al.* 2013; Morales *et al.* 2017). In south-eastern Australia, mito-A birds occur predominantly in more inland and climatically-variable areas with warmer summers, whereas mito-B birds occur in more temperate and climatically-stable coastal areas. Associations of mitochondrial distances with maximum temperature of the warmest month and precipitation of the driest month remain significant after controlling for geographic distance and geographic location (Pavlova *et al.* 2013; Morales *et al.* 2017). Five amino acid replacements in mitochondrially-encoded oxidative phosphorylation Complex I subunits are fixed between the mitolineages. These were inferred to be under positive selection, and predicted to significantly change physio-chemical properties of Complex I and affect the coupling efficiency of the entire pathway (Morales *et al.* 2015; Lamb *et al.* 2018).

It was hypothesized that mito-A birds should have evolved lower mitochondrial respiration rates and more-coupled systems compared to mito-B birds. More coupled-systems would give mito-A birds the benefit of requiring fewer nutrients to synthesise a given quantity of ATP in what is expected to be a nutritionally-poor habitat, while also avoiding excess heat dissipation in a hot climate (Sunnucks *et al.* 2017). Lower rates of respiration would indicate a reduction in the overall cost of living. The difference between mitolineages is expected to be more pronounced in in the function of Complex I, given the signatures of positive selection in some of its subunits.

To test these hypotheses, we developed a novel protocol to measure mitochondrial function of birds in a minimally-invasive way using permeabilized blood cells (Stier *et al.* 2015). This protocol allowed us to measure the contribution of Complex I and proton leak to overall respiration and to estimate mitochondrial coupling. We assessed if mitolineage predicted variation in mitochondrial function in EYR while also testing alternative explanations: short-term acclimation to weather experienced by the wild birds, adaptation to long-term climate of their environment (independent of mitolineage), and variation between EYR such as sex and mass.

Methods

Study Sites and Field Sampling

The study was conducted in Central Victoria, Australia, between May 2017 and March 2018. The four study sites represented a range of climates, based on variation in maximum summer temperature and minimum winter temperature (Table S1). Two sites with contrasting climates (*Pure Hot* and *Pure Cold,* Figure 1) included birds with a single mitolineage (mito-A and mito-B, respectively), and two sites (*Shared Hot* and *Shared Cold*) were co-inhabited by birds of both mitolineages, with documented cases of interbreeding between them (Walters, unpublished Honours thesis, Monash University).

Birds were caught with mist-nets or spring traps, colour-banded, measured, aged, assessed for moult, sampled and released at the site of capture. Cloacal body temperature was measured using a digital thermometer (MicroTherma 2T RET 3, and TW2-1000, Braintree Scientific). Two blood samples (up to 75 µl each) were taken from the brachial vein for genetic and metabolic analyses. Samples for genetic analyses were taken with non-heparinised microcapillary tubes, immediately transferred into 1 ml of absolute ethanol and stored at -20 ˚C after return from the field. Samples for assessment of blood cell mitochondrial function were taken with heparinised microcapillary tubes, transported at ~4 ˚C to a nearby laboratory and assayed within 2 hours of sampling.

Genetic Analyses

Total genomic DNA was extracted from blood using the DNeasy Blood and Tissue extraction kit (QIAGEN, Germany) following manufacturer's instructions. The sex and mitolineage of each individual were determined using sexing PCR and ND2 sequencing (Genbank accession pending), following Pavlova et al. (2013).

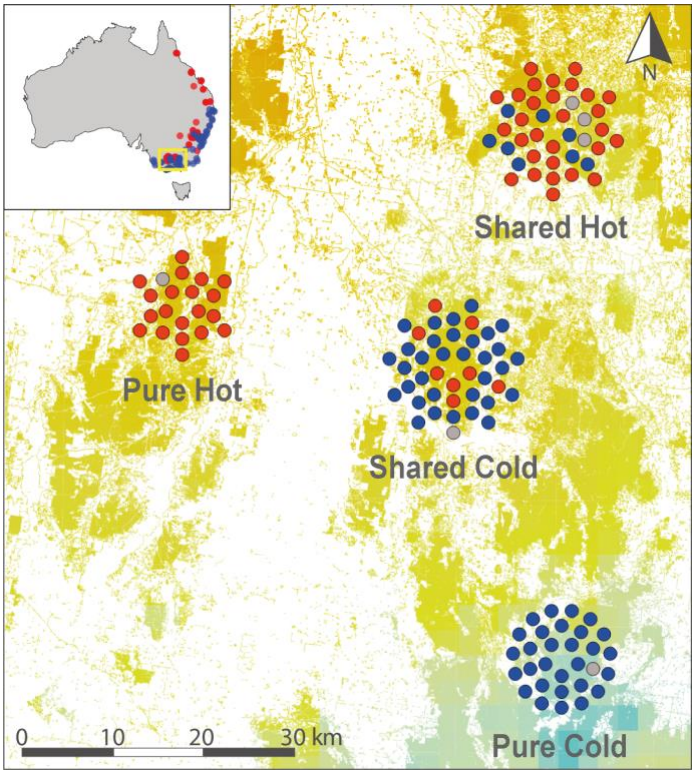

Figure 1: Spatial distribution of mitolineages (red dots- mito-A, blue dots- mito-B, grey – unidentified mitolineage) across the four study sites in Central Victoria, Australia (points displaced for better visibility). The background shading indicates woodland areas coloured according to the 30-year average maximum summer temperatures (from blue (cold, 21 ˚C) to orange (hot, 29 ˚C). White background indicates agricultural or urban non-habitat of EYR. The inset shows distribution of mitolineages throughout Australia and the approximate location of the study area (yellow rectangle). Map was created with QGIS (QGIS Development Team 2019).

Mitochondrial Function

Mitochondrial respiration assay

Because avian red blood cells have functional mitochondria (Stier *et al.* 2013), fresh blood samples taken from wild birds in the field can be used to assay mitochondrial activity (Stier *et al.* 2017). Mitochondrial respiration rates were measured using high-resolution respirometry (*Oxygraph-O2k*, Ororboros, Austria).

Blood cells were isolated from fresh blood by centrifugation and transferred into 1 ml of respiration buffer MiR06 pre-equilibrated at 40 ˚C (Gnaiger *et al.* 2000), for details please see Box S1. The sample was then added to the respirometry chamber which contained 1 ml of MiR06 respiration buffer pre-equilibrated at 40 ˚C (i.e. the average body temperature from EYR cloacal measurements), at which temperature the assay was also conducted. We re-oxygenate the solution by adding small volumes of 200 mM H_2O_2.

We developed a novel substrate-uncoupler-inhibitor titration (SUIT) protocol to quantify the oxygen flux rate (pmol O_2 ml^{-1} sec^{-1}) in the cell suspension at six states (*ROUTINE*, $OXPHOS_{CI}$, $OXPHOS_{CI+CII}$, *LEAK*, *ET*, *ROX*, Figure 2). This assay provides information on mitochondrial energy metabolism for different properties of the oxidative phosphorylation system (Figure 2). In particular, it allowed us to quantify the relative contribution of Complex I to metabolic activity, as well as absolute and relative levels of exothermic proton leak (Gnaiger *et al.* 2000).

After completion of the assay, a 1 ml aliquot of each sample was stored at -20 °C until placed at -80 °C within ten days for long-term storage. Later, this aliquot was used to estimate cellular protein content to control for differences in the amount of sample used in each assay (Stier *et al.* 2017). Protein concentration was quantified in triplicates using a Bicinchoninic Acid (BCA) Protein Assay (G-Biosciences, USA) on aliquots lysed with TWEEN 20 (0.5% v/v). Data extraction from the mitochondrial activity assays and quality control were conducted using a custom R script (Box S2).

Mitochondrial respiration rates

How the mitochondrial respiration rates of the six states are achieved is outlined in detail in Figure 2. The respiration rates of different states provide information on the functioning of different components of the oxidative phosphorylation system. *ROUTINE* respiration is the endogenous respiration rate of intact blood cells when energy demand and other physiological parameters are regulated by the cell (Gnaiger 2014). Cells were subsequently permeabilized to enable the delivery of specific substrates and inhibitors to the mitochondria. $OXPHOS_{CI}$ respiration is the rate at which only Complex I contributes electrons to the system, while $OXPHOS_{CI+CII}$ respiration also includes electron entry at Complex II (Gnaiger 2009). *LEAK* respiration is limited to proton leak along the electro-chemical gradient when ATP synthesis is halted and thus energy is dissipated mostly as heat (Gnaiger 1983). *ET* respiration gives the rate of enzyme activity for complexes I-IV (electron transport system) that is unlimited by ATP synthesis and proton leak (Gnaiger 2009; Gnaiger 2014). During quality control, we determined that *ET* was very variable between reagent batches and it was therefore excluded from further analyses.

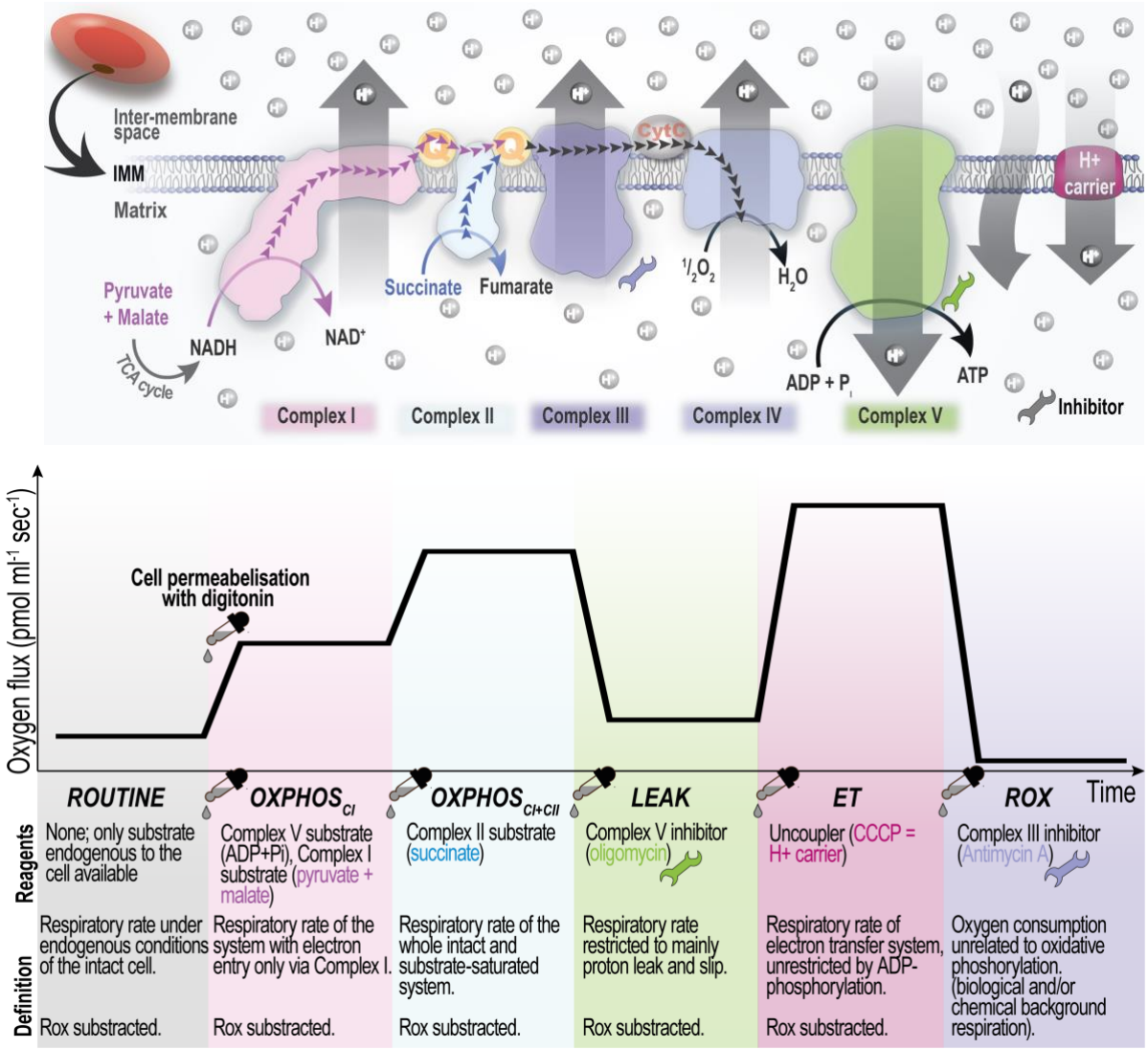

Figure 2: Graphical summary of the high-resolution respirometry substrate-uncoupler-inhibitor-titration (SUIT) protocol developed for measuring oxygen flux in avian blood cells. ***Top panel:*** *Schematic of the oxidative phosphorylation pathway as a reference for the SUIT protocol. Complexes I to IV create the chemiosmotic-proton(H)-gradient across the inner mitochondrial membrane (IMM) by translocating protons from the mitochondrial matrix to the inter-membrane space (arrows across Complexes I, III, IV). Proton translocation is fuelled by electron donors NADH entering via Complex I, and Succinate entering via Complex II. Electrons are transferred between Complexes I and II and Complexes II and III via ubiquinone (Q). The path of electrons (marked as arrows) from both sources join prior to Complex III. Cytochrome c (CytC) transfers electrons to Complex IV, where the electrons reduce oxygen to water. The energy stored in the chemiosmotic-proton(H)-gradient is used by Complex V to fuel phosphorylation of ADP to ATP (arrow across Complex V). Proton leak across IMM happens continuously (warped arrow) and releases metabolic heat. Additionally, inhibitors (small wrench symbols) indicate where inhibitors used in the SUIT protocol act, and the H- carrier in the IMM indicates the function of the uncoupler used in the protocol.* ***Bottom panel:*** *Schematic of the developed SUIT protocol and the corresponding respiration states measured. First, ROUTINE respiration rate of intact cells, reflecting the endogenous respiration of cells with their own substrates and ADP/ATP demand was measured. Cells were then permeabilized with digitonin (20.3 µM) to enable supply of substrates*

and inhibitors to the mitochondria, and a saturating amount of ADP was added (1.25 mM). OXPHOS$_{CI}$ respiration rate fuelled by Complex I was measured after addition of its substrates Malate and Pyruvate (2 mM and 5 mM, respectively). OXPHOS$_{CI+II}$ respiration rate fuelled by Complexes I and II was then measured by providing Complex II's substrate Succinate (12.5 mM). LEAK respiration rate linked to proton leak was subsequently assessed by inhibiting Complex IV with Oligomycin (2.5 μM). ET respiration rate of the electron transport system unlimited by the rate of ATP synthesis was measured after the titration of uncoupler (Carbonyl cyanide m-chlorophenyl hydrazine, 0.5 μM to 2.5 μM). ROX, the residual oxygen consumption not related to oxidative phosphorylation was measured by inhibiting Complex III with Antimycin A (2.5 μM), and subtracted from other measurements.

Flux control ratios

Three additional response variables, referred to as flux control ratios, were calculated from measurements of mitochondrial respiration rates. Being ratios of respiration rates, they allow comparisons of mitochondrial function independently of normalization methods (i.e. per cell number, protein concentration, mitochondrial density) (Gnaiger *et al.* unpublished ; Gnaiger 2014)and inform on coupling state and relative contribution of specific parts of the oxidative phosphorylation system. The ratio of *LEAK:OXPHOS$_{CI}$* respiration normalizes *OXPHOS$_{CI}$*. The ratio of *LEAK:OXPHOS$_{CI+CII}$* respiration reflects the proportion of mitochondrial activity releasing heat mainly via proton leak, thus is a parameter estimating mitochondrial coupling (Gnaiger 1983, 2014; Gnaiger *et al.* 2015). The ratio of *OXPHOS$_{CI}$:OXPHOS$_{CI+CII}$* reflects relative contribution of Complex I to overall respiration.

We analysed flux control ratios and respiration rates separately because of their different biological meanings and different units. Mitochondrial respiration rates provide information about the capacities of oxidative phosphorylation, but here do not allow to differentiate between effects of different mitochondrial density and varying protein activities between individuals. Flux control ratios on the other hand, provide information on properties of the oxidative phosphorylation that are directly linked to the biochemical components of the mitochondria, independent of mitochondrial quantity.

Predictors: Climate, Weather, and Individual Variables

We included climate, weather and individual-specific variables in our analyses of mitochondrial function. Climate variables were included to assess long-term climatic effects on mitochondrial function that likely will indicate climate adaptation. Some of these variables will be confounded with mitolineage distribution (Figure 3). However, others might not be confounded with

mitolineage distribution and thus allow us to infer alternative climatic drivers of variation in mitochondrial function. Weather variables were included to asses short-term acclimation to the environment.

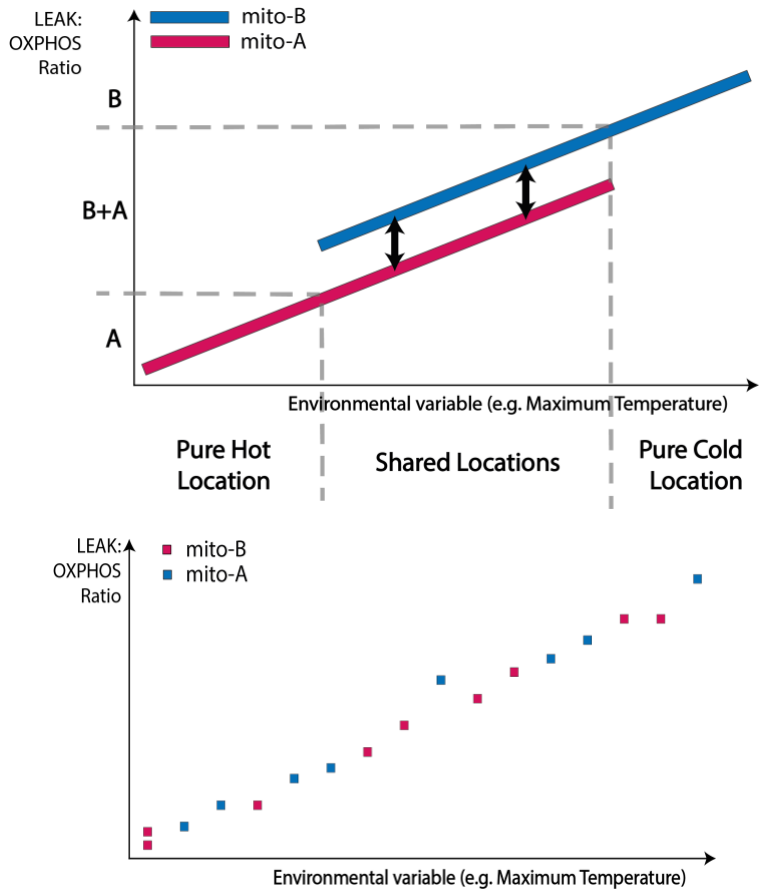

Figure 3: Scenarios of climate association of mitolineage distribution. **Top panel:** Distributions of both lineages are correlated with a particular climate variable. In the central range of this variable studied across our four study sites are the shared locations. If mitolineage conveys difference in coupling efficiency (LEAK:OXPHOS ratio) the individuals of different lineages should differ (arrows) in the shared locations (as long as within-site variation of climate is small, which is expected). Coupling efficiency should display three distinct ranges (y-axis) corresponding to the two pure and the shared sites. **Bottom panel:** No association of mitolineage distribution and a particular climate variable. Coupling efficiency increases with the climate variable, but is independent of mitolineage.

Based on the limited knowledge of mitochondrial function in wild birds we collected large numbers of variables for each category to test their relevance. We had no *a priori* expectation of the climate and weather conditions that would be important predictors of mitochondrial function. All of the available climate, weather, and individual variables included might reasonably be hypothesised to have an influence on mitochondrial function. Rather than arbitrarily or subjectively selecting variables for inclusion, we adopted a newly-developed Bayesian Variable Selection for Multivariate data approach for reducing large numbers of variables to smaller sets in an unbiased fashion (Liquet *et al.* 2017).

Climate variables included monthly and annual 30-year averages of summary statistics of rainfall, temperature, relative humidity, evaporation, evapotranspiration and solar exposure (n=142, Table S2). Daily weather variables were summarised for a range of time periods prior to capture as estimates of the conditions EYR were exposed to just prior to metabolic assessments. We did not have a *a priori* expectation for the relevant acclimation time thus tested different durations (t = 3, 7, 14, 21, 28 and 56 days prior to capture, n= 123, Table S3). Climate and weather data were sourced from the Australian Bureau of Meteorology (Commonwealth of Australia 2018b, 2018a). Data for bird capture locations were extracted from grids (resolution of 5 x 5 km or higher) with the *Geographic Data Analysis and Modelling* package *raster* (Hijmans 2018) in *R v.3.5.1* (R Core Team 2018) and *RStudio v.1.1.414 (RStudio Team 2016)*.

Because an individual's physical characteristics and condition can alter its cellular processes (Brand *et al.* 2003), we included in our analyses of cellular metabolic activity individual variables encompassing characteristics of EYR (such as sex, mass and body condition) at the time of capture, along with temporal and spatial information (Table S4, Box S3).

Modelling mitochondrial function

To test whether the spatial scale of the analyses, and thus the range of environmental variation included, can impact our inferences, we analysed both sets of response variables (mitochondrial respiration rates and flux control ratios) independently on a large and a small special scale. The large-scale dataset comprised all four study sites. The small-scale dataset only included the two shared sites. Because the latter comprised locations where mito-A and mito-B mitolineages co-occur in very similar environments, this dataset should enable us to distinguish mitolineage- and environmental effects on metabolic activity. Furthermore, the small-scale dataset contained two sites with slightly differing climate, but a strong putatively climate-

associated skew in mitolineage composition of the population (Table S1, Figure 1). At the Shared Hot site 69 % of individuals were of the mitolineage related to hotter climates (mito-A), whereas at the Shared Cold site only 25 % were of this mitolineage. By contrasting these shared sites, we can assess each mitolineage in an environment where they are a 'local' and where they are an 'immigrant'. This is the closest approach testing local adaptation of the two mitolineages considering ethical, logistic and regulatory constraints.

Our aim was to determine the relevance of mitolineage to mitochondrial function in EYR, and individual, climatic and weather variation encapsulated in 296 predictor variables. We achieved this via a cascade of analyses steps (Figure 4). First we replaced clusters of highly-correlated environmental variables ($r>0.9$) with one randomly-chosen representative variable. Subsequently, we reduced the number of remaining predictor variables to include in the following step by using a novel tool for variable selection (MBSGS), that utilises the group structure of the predictor variables and multivariate-nature of our data (Liquet *et al.* 2017; Liquet and Sutton 2017) (Figure 4. bottom left, Box S4.1). Following variable reduction, we created multivariate models of the mitochondrial respiration rates and flux control ratios separately. We created one model for every possible combination of one to three predictor variables (the multivariate models of mitochondrial respiration rates always included an additional technical variable: protein concentration) using MuMIn (Bartoń 2018). This approach allowed us to assess the effect of mitolineage with and without the possible other driving forces (in particular climate) that might interfere with the signal from mitolineage. Each model was rated with a quality score (AICc), based on which the models for consideration in the last step were chosen (Figure 4 top right, Box S4.2). For each model short-listed in the previous step, the effect of each predictor variable on each response variable was assessed. Subsequently, these individual effects were summarised for all models short-listed, which formed the basis for inferring conclusions. Extending our assessment to include models with a relatively large drop in quality scores and assessing parts of the model instead of only the whole allowed us to pick up even weak trends, which might occur when predictor variables are competing with each other due to collinearity (Figure 4 bottom right, Box S4.3). Summarising across models also highly reduces the risk of focussing on false positives that might arise by chance in a particular combination of predictor variables. Only if a particular variable is consistently increasing the quality of a model did we consider it relevant. To do this we estimated dominance (proportion showing how many times a particular predictor variable had a significant effect on a multivariate response in all best models). We only focussed on models

with high dominance in our interpretations, disregarding variables with low dominance as potential Type I error.

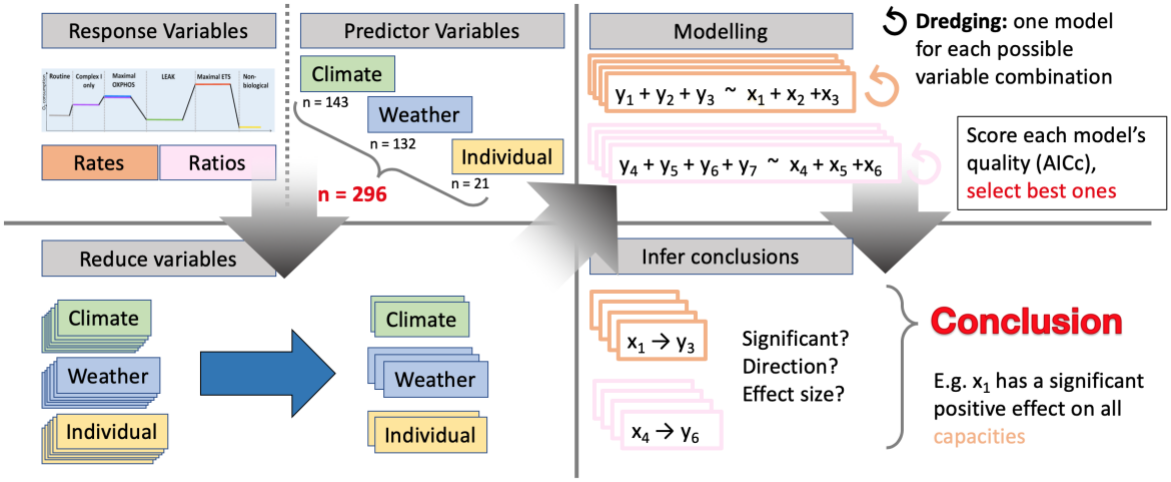

Figure 4: Graphical summary of analyses. ***Top left:*** *Step 1 - Data collection. Mitochondrial function was assessed in wild EYR by obtaining mitochondrial respiration rates and flux control ratios. Rates and ratios were collected as response variables for models in step 3, while climate, weather and individual variables were collected as possible predictor variables in these models.* ***Bottom Left:*** *Step 2 – Reduction of number of predictor variables. First, to avoid overrepresentation of highly-correlated environmental variables, we assessed correlations for all weather, all climate and all individual variables separately and randomly chose a single variable from clusters of highly correlated ones (r>0.9). Subsequently, we used Multivariate Bayesian Sparse Group Selection with Spike and Slab (MBSGS) (Liquet et al. 2017) to reduce the number of predictor variables further. We always retained mitolineage, sex, season, age and study site in the predictor variable set for the next step as we wanted to estimate their effects.* ***Top right:*** *Step 3 – Modelling. We created and assessed the quality of multivariate models of the rates and ratios separately. Each model is rated with a quality score (AICc), based on which the models for consideration in the last step are chosen (ΔAICc <10).* ***Bottom Right:*** *Step 4 – Infer conclusions. For each model short-listed in the previous step, the effect of each predictor variable on each response variable is assessed. Subsequently, these individual effects are summarised for all models short-listed, which forms the basis for inferring conclusions.*

Results

Across all EYR samples (n=127) the respiration rate of intact cells (*ROUTINE*) averaged 6.80 (SD=3.80) pmol O_2 mg^{-1} sec^{-1} (Figure S1). *LEAK* respiration rate (mean =10.00, SD=3.82) represented ~25% of the respiration of the whole substrate-saturated system ($OXPHOS_{CI+CII}$, mean 43.00, SD = 21.00). Complex I-linked respiration comprised ~60% that of the whole system ($OXPHOS_{CI}$ mean = 25.04, SD=10.86).

Strongest predictors of mitochondrial function

We created and assessed several hundred models for each dataset (Table S6.1-4). The best models (ΔAICc <10) for the mitochondrial respiration rates included only weather variables as predictors (Figure 5). In contrast, the flux control ratio models of the large-scale dataset (including all four study sites) predominantly included climate variables (Figure 5).

Across small- and large-scale models of mitochondrial respiration rates and flux control ratios short-term estimates of vapour pressure were the strongest predictors (Table S5, Figure S2). Across all sites, average vapour pressure at 3 pm during the 56 days prior to capture of the individual bird is positively associated with heat-linked respiration (*LEAK, average posterior mean = 0.24*). In contrast, the lowest recorded vapour pressure at 3 pm in the 56 days prior to capture is negatively associated with all respiration rates. In 81% of all best models, this effect of lowest recorded vapour pressure is significant for Complex I linked respiration, making it the most common significant term across all datasets.

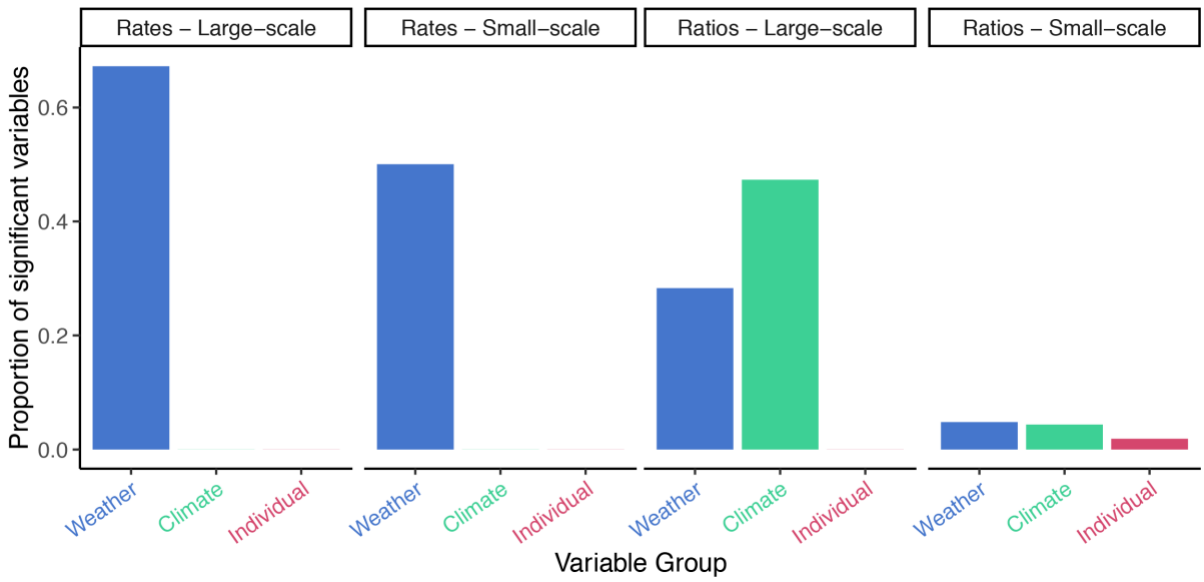

Figure 5: Summary of model response variable groups from the best models (ΔAICc <10) for mitochondrial rates (rates) and flux control ratios (ratios) measured at large and small spatial scales. Bars indicate the proportion of models in which response variables of a certain group were present and significant out of the total models summarised.

Mitolineage as a predictor of mitochondrial function

We assessed the association of mitolineage with mitochondrial function alongside climate and weather variables because mitolineage and climate are confounded on a large spatial scale. Variation in mitochondrial respiration ($OXPHOS_{CI-CII}$) and Complex I-linked respiration ($OXPHOS_{CI}$) across all study sites (large-scale) was not explained by the mitolineage of the individual (Table 1, Figure 6A). Instead, a weather variable (minimal vapour pressure during the 56 days prior to capture) was the single best predictor for all mitochondrial respiration rates (Figure S2), with respiration rates increasing as minimum vapour pressure drops (Figure 7). Similarly, when restricting the analysis to the shared sites only, which have very similar climates, mitolineage did not explain mitochondrial respiration rates, but weather did (Table 1, Figure 6, 7). Only two predictors were significant for this set, both exhibiting a positive effect on respiration rates: number of days without rain in the 7 days prior capture and the average vapour pressure (during the 56 days prior to capture).

Mitolineage also did not significantly explain variation in mitochondrial coupling ($LEAK{:}OXPHOS_{CI-CII}$) or in the contribution of Complex I to overall respiration ($OXPHOS_{CI}{:}OXPHOS_{CI-CII}$) on either the small or the larger spatial scale (Table 1, Figure 6). The strongest test in our dataset is between individuals of different mitolineage in the shared site. We expected to see a difference between Complex I related and coupling related respiration parameters – at least after accounting for acclimation to different conditions. When directly contrasting the best mitolineage-containing models to the best overall model for each set, mitolineage-containing models are of substantially lower quality (>18 ΔAIC). Most importantly, mitolineage effects are not significant in the best mitolineage containing models. This holds true even after accounting for acclimation for the large-scale dataset on mitochondrial respiration rates. Instead, variation in flux control ratios were best predicted by climate variables (Figure 5). The single best predictor of variation in flux control ratios across all sites was historical evapotranspiration (May), with an increase in coupling and relative Complex I contribution with increasing evapotranspiration (Figure 7). However, the different study locations did not correspond to evapotranspiration, mainly because variation in the Pure-Hot site spanned the entire range of evapotranspiration estimates recorded (Figure S3). Models of

only the shared sites (small-scale) were generally not good at predicting the variation found in all flux control ratios, as the few significant predictors found for those models only explained one response variable at a time (Figure 7, Table S5).

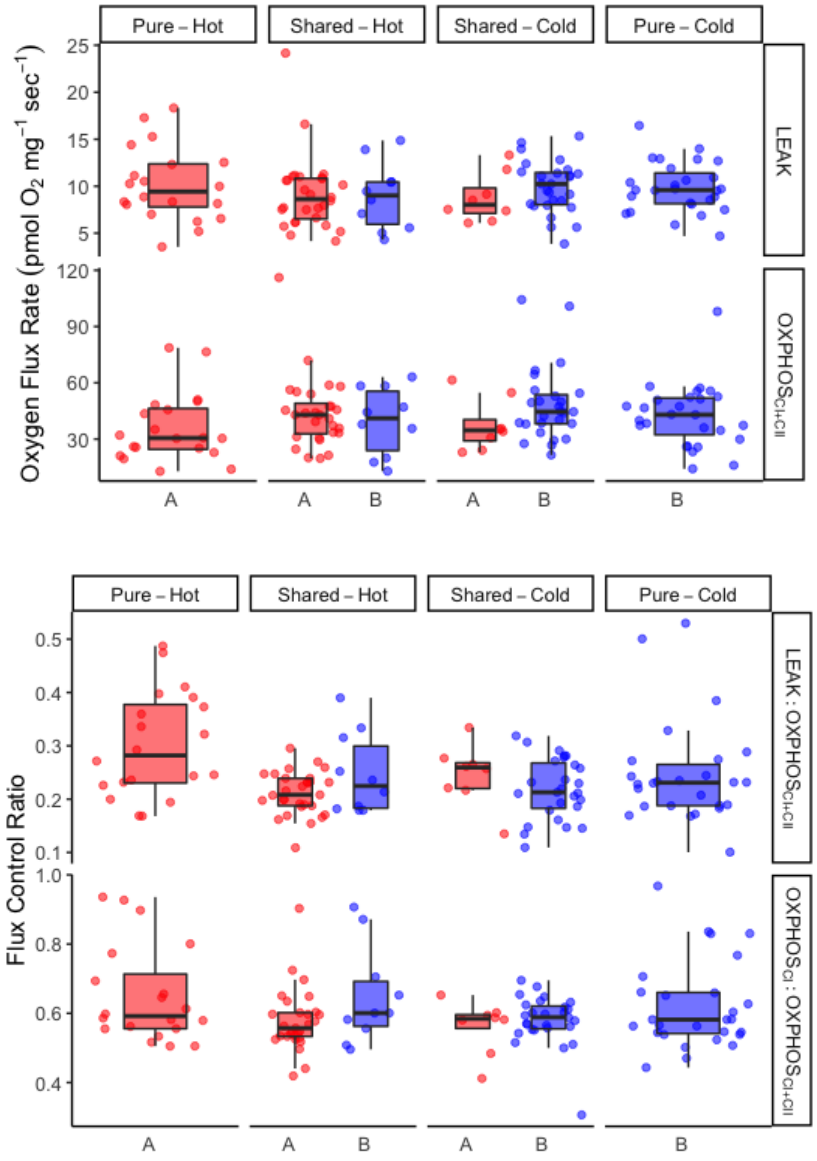

Figure 6: Mitochondrial respiration rates and flux control ratios as measures of mitochondrial function in wild EYR (n=127), showing the raw data points, and box and whisker plots illustrating quartile-ranges for the four study sites. Data was split into study sites and sex or mitolineage to visualise the differences of mitochondrial function between these groups. ***Upper panel:*** *Mitochondrial respiration rates split by mitolineage and study site.* ***lower panel:*** *Flux control ratios split by mitolineage and study site.*

Table 1: Best models of predictors of mitochondrial respiration rates and flux control ratios, at each of two spatial scales, with comparisons to the best models that included mitolineage as a term. The large-scale datasets included all four study sites, small-scale ones included only the sites with similar climates where both mitolineages co-exist.

Scale-Response	Best Model overall	AICc	Best Model that included mitolineage	AICc	Mitolineage significant?	ΔAICc
Large-Rates	~ Min. Vapour Pressure (56D) + Protein conc.	793.81	~ Mitolineage + Min. Vapour Pressure (56D) + Protein conc.	827.64	no	33.83
Small-Rates	~ Days without Rain (7D) + Protein conc.	491.51	~ Mitolineage + Protein conc.	541.69	no	50.18
Large-Ratios	~ Historical Evapotranspiration (May)	698.00	~ Mitolineage + Historical Evapotranspiration (May)	723.87	no	25.87
Small-Ratios	~ Historical Relative Humidity (May)	475.60	~ Mitolineage + Beak	494.30	no	18.70

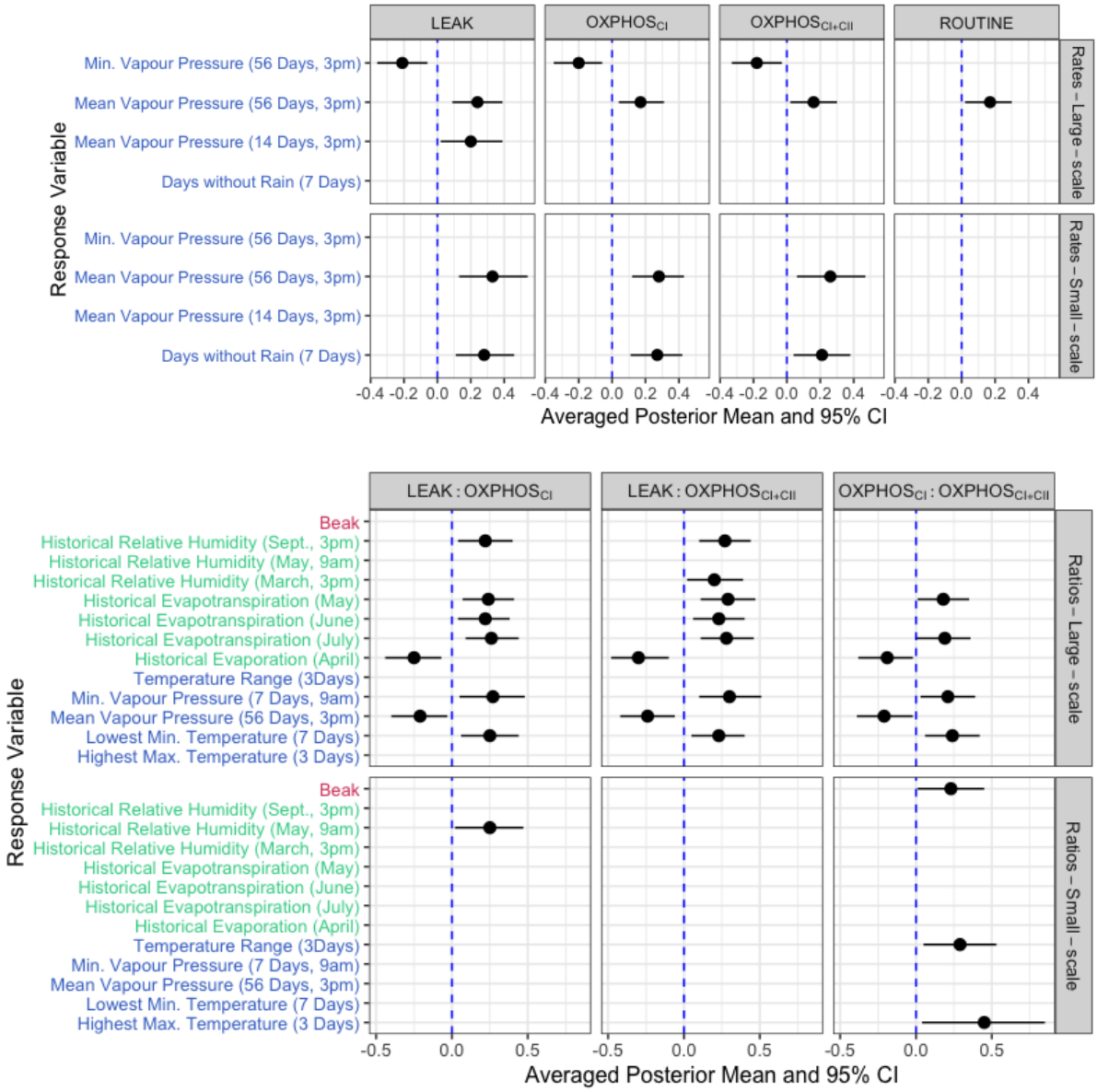

Figure 7: Summary of the effect of all significant predictor variables in the best models (ΔAICc <10) to each response variable for small and large spatial scale of mitochondrial respiration rate models ***(top panel)*** *and models and flux control ratio models* ***(bottom panel)****. Points indicate the posterior mean and lines indicate the corresponding 95% confidence interval. Estimates were averaged over all significant occurrences of the effect across the best models (Table S4). The effect sizes are given in standard deviations of the predictor to the response variable. Colour of the predictor variable indicates its membership to, red: individual variables, blue: weather variables, and green: climate variables.*

Humidity-related environmental variables as predictors of mitochondrial function

Climate and weather variables that explain variation in mitochondrial function were related to water availability and humidity rather than temperature (Figure 8). Variation in mitochondrial respiration (*OXPHOS*$_{CI+CII}$), heat-linked respiration (*LEAK*), and Complex I linked respiration (*OXPHOS*$_{CI}$) were explained only by short-term variation in humidity or rainfall (Figure S2, Table S5). There was a strong trend of respiration rates increasing with average vapour pressure (Figure 7). Variation in coupling (*LEAK:OXPHOS*$_{CI+CII}$) and the contribution of Complex I to overall respiration (*OXPHOS*$_{CI}$ *:OXPHOS*$_{CI+CII}$) across all sites was predominately explained by both long- and short term variation in humidity: short-term vapour pressure, long-term averages of relative humidity, evaporation, and evapotranspiration (Figure S2, Table S5). Only one temperature-related variable was present in the best models for this set: flux control ratios increased with an increase in the lowest minimum temperature experienced by the bird in the week prior to capture (Figure 7).

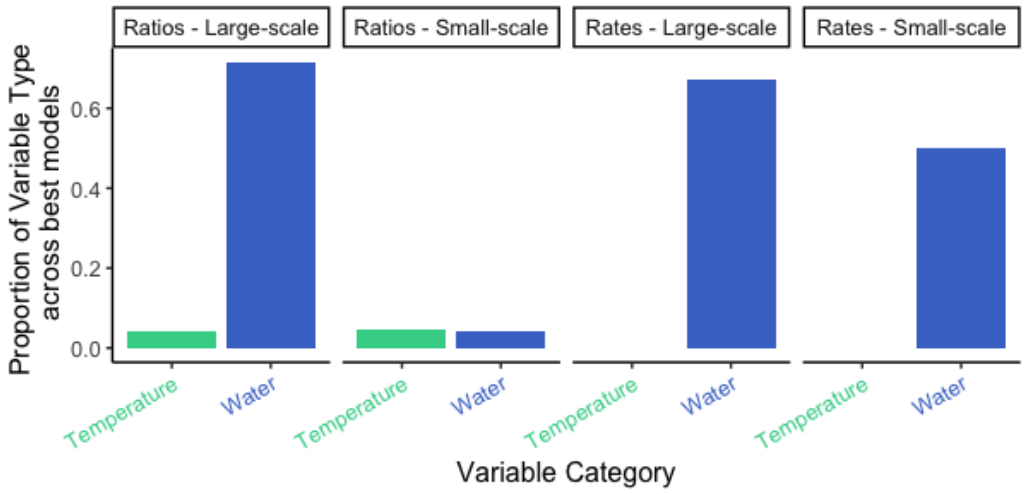

Figure 8: Temperature and water-related significant predictor variables in the best models (ΔAICc < 10) of mitochondrial respiration rates (rates) and flux control ratios (ratios) measured at large and small spatial scales. Bars indicate the proportion of models in which variables of a certain class were present and significant out of all models summarised.

Seasonal climate as a predictor of mitochondrial function

The climate variables that best explain the variation in mitochondrial coupling (*LEAK:OXPHOS*$_{CI-CII}$) and the contribution of Complex I to overall respiration (*OXPHOS*$_{CI}$ *:OXPHOS*$_{CI-CII}$) are the long-term averages of climatic conditions during cooler months (Figure 9, Table S5, Table S2). In particular, the models comprising all study sites were dominated by water-related climate variables in the coolest months (evapotranspiration in May/June/July, Figure S2, Figure 7). Climate variables of the hotter months had significant effects on flux control ratios in fewer models, and involved more direct measures of humidity (relative humidity and evaporation, Figure S2, Figure 7).

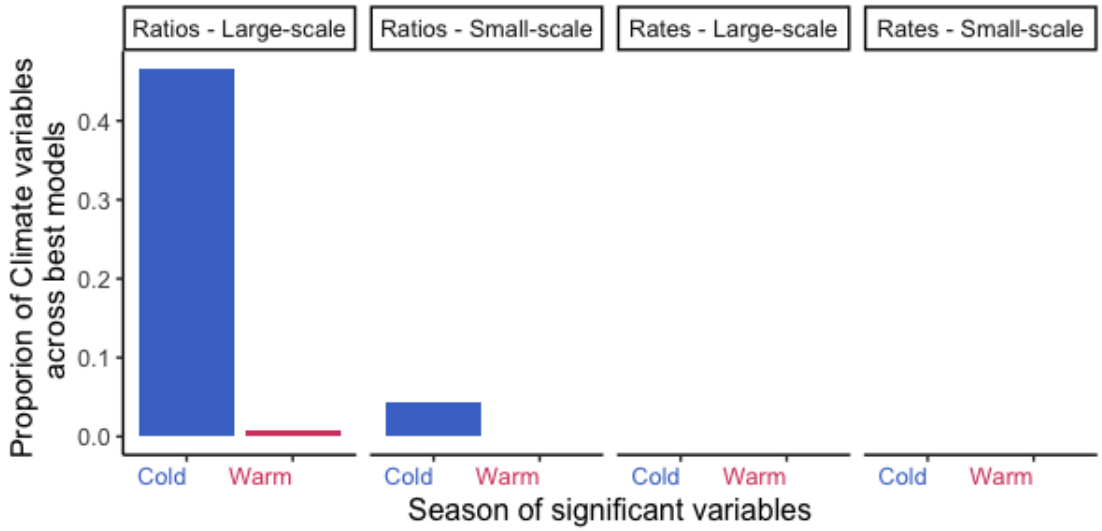

Figure 9: Climate variables significant in the best models (ΔAICc < 10) and classified into cold and warm seasons and split by dataset. Seasons were based on average temperatures being <15 °C or >15 °C, respectively. Cold season: Included climate variables that were summarized by month for the months falling between and including May – October. Warm season: Included climate variables that were summarized by month for the months falling between and including November – April.

Discussion

Mitolineages do not significantly explain variation in mitochondrial activity

Despite our expectations, we did not detect a significant association of mitolineage with mitochondrial respiration rates, coupling of the OXPHOS system, and contribution of Complex I. Even if no other climate or weather variables were considered, mitolineage did not explain the variation in mitochondrial function across all sites or in the shared sites. Similar to studies that compared diverged mitolineages after acclimation to common conditions (Ballard and Melvin 2010; Oellermann *et al.* 2012; Baris *et al.* 2016a; Laporte *et al.* 2016; Martinez *et al.* 2016; Wolff *et al.* 2016a; Chung *et al.* 2017; Leo *et al.* 2017) we expected that at least in the shared sites - where climate is much more similar than in the pure sites - mitochondrial activity would be affected by mitolineage. However, an important difference to these previous studies is that birds in the shared sites experience an intermediate climate, to which neither mitolineage might be particularly well adapted. In the above-mentioned studies, acclimation was usually to temperatures typical for each mitolineage, not their intermediate. We tried to approximate conditions in these in-situ studies by choosing two shared sites, one more allied in climate to that of one mitolineage and the other site to the other mitolineage. Consistent with this, the hotter site had approximately twice as many mito-A birds as mito-B, and the reverse ratio was true for the colder shared site. However, ΔAICc for models containing the interaction term *study site × mitolineage* was always greater than 10, and therefore no models with this interaction term were retained in the model set used for conclusion averaging.

In our EYR study system, there is evidence that some nuclear encoded OXPHOS genes co-evolved with the mitogenome (Morales *et al.* 2018). Accordingly, not accounting for variation in these genes and degree of co-inheritance of co-evolved alleles with a mitochondrial genome (i.e. mito-nuclear genotype) could explain the absence of a mitolineage effect in the hybrid zone. A ~15.4 megabase nuclear genomic region with strong overrepresentation of OXPHOS genes and very high linkage disequilibrium is differentiated between the two mitolineages (Morales *et al.* 2018). The high linkage disequilibrium maintains a set of alleles that are almost always inherited together, and also co-inherited with the corresponding mitolineage. In hybrid zones however, co-evolved mito-nuclear alleles of different loci can be separated when birds of different mitolineages produce viable offspring, particularly beyond the first generation. Thus, the mitochondrial function of hybrid birds with scrambled allele combinations might differ

from that of birds from pure sites. It is also possible that natural selection on these genomic arrangements has operated differently in the hybrid zone than in the 'pure' sites.

Humidity-related climate parameters are associated with mitochondrial function

Variation in mitochondrial function seems to be explained predominantly by long-term climate variables and short-term weather variables related to water. The mitochondrial flux control ratios were most affected by variables of long-term climatic averages related to water. In contrast, mitochondrial respiration rates were most commonly affected by short-term weather variables related to humidity and rainfall. Thus, our findings do not align with the common observation that the main driver of avian metabolism is ambient temperature (Tieleman and Williams 2000b; McKechnie 2008; McKechnie and Swanson 2010; Stager *et al.* 2015; Stager *et al.* 2016; Albright *et al.* 2017), but are in accordance with the growing body of evidence showing that water is an important factor shaping avian metabolism (Tieleman *et al.* 2003a; White *et al.* 2007a; Albright *et al.* 2009; Smit and McKechnie 2015; Noakes *et al.* 2016).

Mitochondrial coupling (*LEAK:OXPHOS*$_{CI\text{-}CIII}$), and thus relative heat production was higher, and mitochondrial efficiency lower, in areas with higher evapotranspiration, but did not frequently show strong correlations with other more direct measures of humidity, such as relative humidity. Because evapotranspiration depends on soil, precipitation, temperature, vegetation, solar radiation and wind (Allen and Food and Agriculture Organization of the United Nations. 1998), a more complex relationship may exist between relative heat production/efficiency and environmental conditions. To isolate these effects, more detailed studies into the relationships of evapotranspiration to other biotic and abiotic factors relevant to EYR are required. For example, it is possible that evapotranspiration affects food resources available to EYR, and thus, it could be more beneficial for EYR to have more efficient mitochondrial system at these sites (Monternier *et al.* 2014; Holmbeck and Rand 2015).

Winter as season of critical climatic stress

The mitochondrial flux control ratios of EYR show signs of adaptation to winter climate. Annual averages of climatic conditions do not predict mitochondrial function well: they were not represented in the short-listed best models. Furthermore, variables of warmer months were only relevant in few models. The strongest patterns were observed for mitochondrial coupling (*LEAK:OXPHOS*$_{CI\text{-}CIII}$), and these were associated with evapotranspiration in the coolest

months. Although limited, this provides evidence for winter being the season of critical climatic/environmental stress shaping the evolution of mitochondrial function in EYR. Winter has also been identified as the season of critical thermal stress in other bird species (Danner and Greenberg 2015; Stager *et al.* 2016; Wang *et al.* 2016; Cheviron and Swanson 2017; Wehenkel *et al.* 2017). However, winter temperatures were not associated with mitochondrial coupling in EYR. Instead evapotranspiration in the coolest months was positively associated with mitochondrial coupling (*LEAK:OXPHOS*$_{CI+CII}$). Given that evapotranspiration is positively associated with primary production (Brümmer *et al.* 2012), reduced food availability for EYR in winter (via lower food availability for prey species) may be the main stressor for EYR in winter.

Conclusion

In this study we tested if the diverged mitolineages in the Eastern Yellow robin, which are expected to harbour functional differences in Complex I, affect the birds' mitochondrial function. However, our analysis did not indicate such a difference between the two mitolineages. Instead, our analysis indicated that environmental variables related to humidity (and not temperature - a commonly inferred predictor of metabolic variation) could be the main driver of variation in mitochondrial function in EYR. In particular, long-term climate variation in humidity during the coldest season was predicting mitochondrial function well. Finally, we provide evidence that mitochondrial respiration rates respond to short-term weather conditions experienced, whereas mitochondrial flux control ratios could be shaped by long-term adaptation to climate.

Acknowledgements

The authors would like to thank the many volunteers that enabled us to complete the challenging field work. In particular, we would like to thank Nevil Amos and Pete Collins without whom this project would have not been possible, and the most dedicated volunteers Sara Petrovic and Jade Ronke. We would also like to acknowledge the great work of field assistants Steven Bianchi, Thomas Richard and Lana Austin. We extend our gratitude to researchers who have been crucial to understanding this system, in particular Hernán Morales, Lynna Kvistad, Han Ming Gan, and Gabriel Low.

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Appendix

Figures

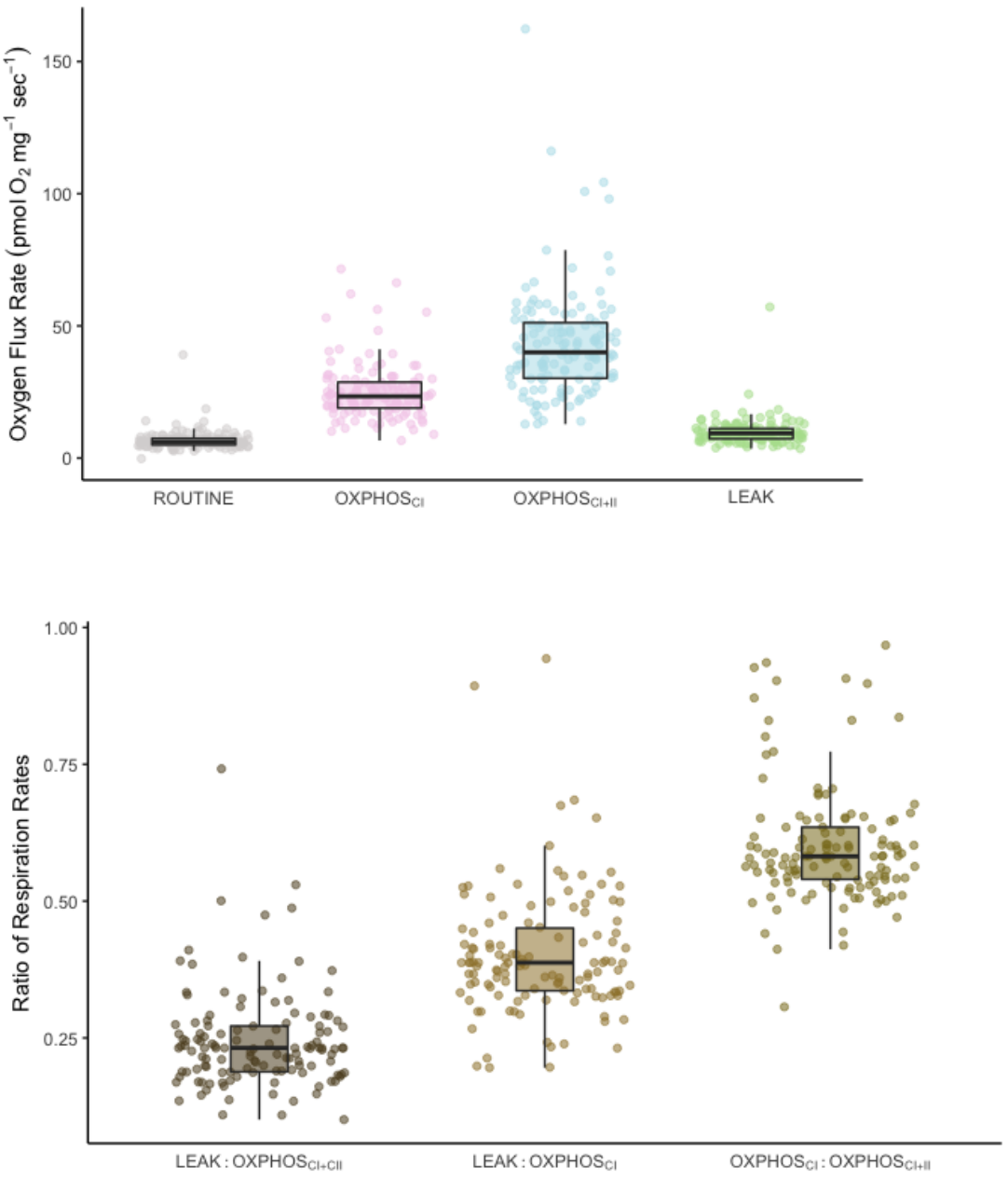

Figure S1: Mitochondrial function measurements of wild Eastern Yellow Robin (n=127), showing the raw data points (randomly scattered horizontally) and box and whisker plots illustrating interquartile-ranges. ***Top panel:*** *Mitochondrial respiration rates at states: ROUTINE, OXPHOS$_{CI}$, OXPHOS$_{CI+CII}$, and LEAK, given as oxygen flux rates measured via high resolution-respirometry of fresh blood cells. Rates are given in pmol of oxygen turned over per second and normalized for protein content of the sample in mg.* ***Bottom panel:*** *Ratios of rates of respiration; LEAK-to-OXPHOS$_{CI}$, LEAK-to-OXPHOS$_{CI+CII}$, and OXPHOS$_{CI}$-to-OXPHOS$_{CI+CII}$.*

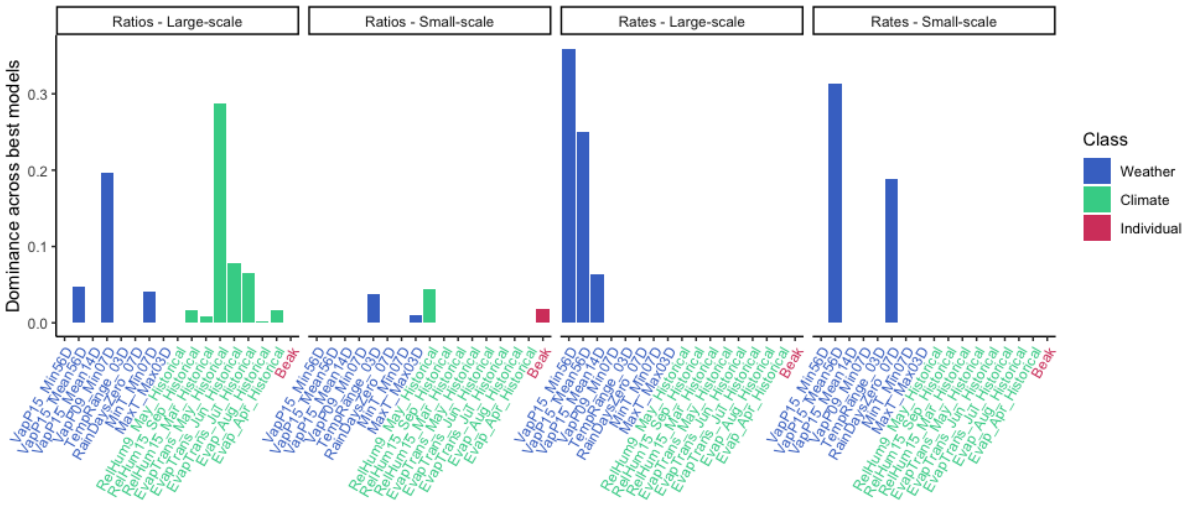

Figure S2: Summary of model terms from the best models (ΔAICc <10) for rates and ratios measured at large and small spatial scales. Bars indicate the proportion of models in which a given term was present and significant out of the total models summarised.

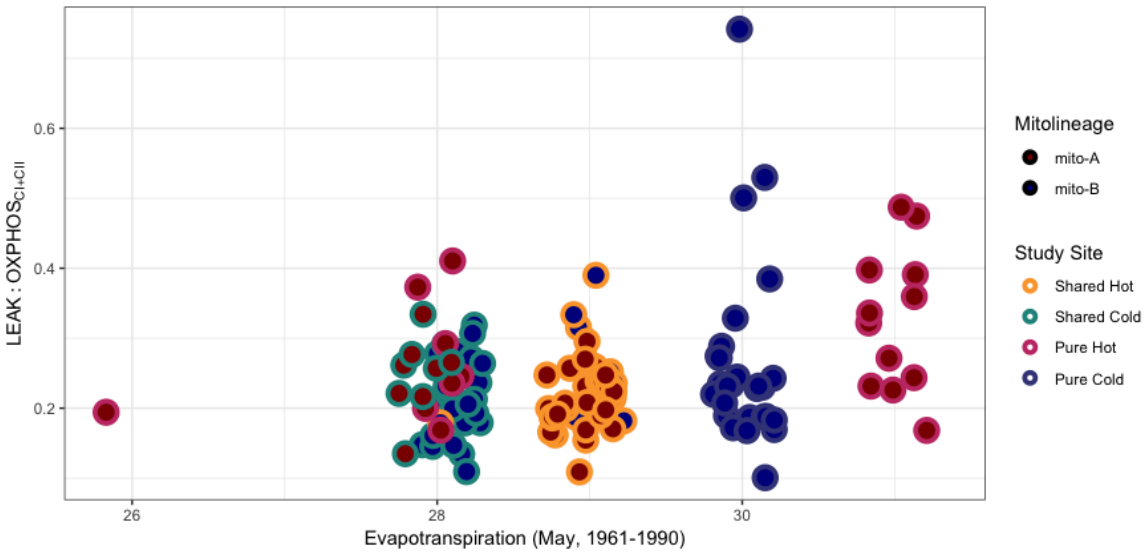

Figure S3: Estimates of coupling efficiency (LEAK:OXPHOS$_{CI-CII}$) of Eastern Yellow Robin for all study sites and mitolineages explained by historical Evapotranspiration in May – the strongest predictor of coupling control ratios across all sites. Small random jitter applied for visibility of data points. Evapotranspiration rates differ largely consistently between sites, except for the Pure Hot site, which is the most variable (containing both the highest and the lowest recorded rates).

Boxes

Box S1: Blood separation into cells and plasma via centrifugation

A six-step procedure was used to prepare blood cells from whole blood immediately before commencement of the assay: 1) the sample (20-75 µl) was spun at ≥1500 g for ≥5 minutes, 2) the plasma fraction was removed, 3) the blood cells were resuspended in 1 ml cold PBS, 4) the suspension was spun at ≥500 g for ≥3 minutes, 5) the supernatant was removed, 6) the pelleted blood cells were resuspended in 1 ml of respiration buffer MiR06 pre-equilibrated at 40 °C (0.5 mM EGTA, 3 mM $MgCl_2 \bullet 6H_2O$, 60 mM K-lactobionic acid, 20 mM taurine, 10 mM KH_2PO_4, 20 mM HEPES, 110 mM D-Sucrose, 0.1% (w/v) essentially fatty acid free BSA, 280 u/ml Catalase, pH 7.1 (Gnaiger *et al.* 2000).

We did not remove the white blood cells due to the small volume of blood available, but it has been shown previously in captive zebra finches that they make a negligible contribution to overall respiration rate measured from blood cells, in accordance with their very low abundance relative to red blood cells (Stier *et al.* 2013).

Box S2: Custom R-script for data extraction and QC of mitochondrial respiration rates from high-resolution respirometry with O2k-oxygraph

The script uses the exported DatLab files to restructure and extract the data. For each state, average rates were calculated from a stable one-minute sample. Additional measures of quality, such as rate of decline were also calculated. Rates that did not meet quality control checks and could be related to technical issues were removed.

Box S3: Additional variables of body condition as predictors of individual differences in metabolic phenotype

All additional estimates were based on a larger dataset (n=610), which included birds captured in the four study sites that were not assessed for mitochondrial activity. Two *Scaled Mass Indices* were calculated based on Peig et al. 2009 (Peig and Green 2009), using tarsus and head-bill length, respectively, to correct the recorded mass for size. We calculated *predicted mass*, which predicted mass based on a linear relationship of a size score of five body part measures (tarsus, head-bill, wing and tail length and bill depth, scored by five inter-quantile-ranges) to the recorded mass. This measure gives the average mass expected for an EYR of a certain size. *Residual mass* was derived from the residuals of the same linear model. Furthermore, we included two measures of *residual condition* by scaling mass to tarsus (mass $tarsus_{-1}$) and head-bill length (mass $bill_{-1}$). Finally, the individual response variable set comprised total of 21 parameters (Table S4).

Box S4

S4.1: Variable reduction with bayesian variable selection for multivariate data

To avoid overrepresentation of highly-correlated environmental variables, we assessed correlation for all weather, all climate and all individual variables separately and randomly chose a single variable from clusters of highly correlated ones ($r>0.9$). To reduce the still large number of possible predictors we used Multivariate Bayesian Sparse Group Selection with Spike and Slab (MBSGS) (Liquet *et al.* 2017), implemented in the R package MBSGS (Liquet and Sutton 2017) to select a subset of variables important to variation in mitochondrial function. We grouped all predictor variables into the three units outlined above: climate, weather and individual (Table S1, S2, S3). By utilising such grouping of predictor variables, MBSGS increases its power to detect associations between multivariate responses and many predictors, compared to other Spike-and-Slab regression methods for variable selection (Liquet *et al.* 2017). Two hundred independent repetitions were run to account for the stochasticity of the Markov Chain Monte Carlo (MCMC) approach, with 30,000 iterations including 15,000 iterations burn-in.

S4.2: Bayesian modelling and model selection

Variables selected by MBSGS were used as predictors of metabolic responses in Bayesian models. Seven additional variables were used as predictors, as they were of biological (*mitolineage, sex, season, study site, age,* Table S3) and technical relevance (*date, trip ID,* Table S3). We also included six interactions that may reflect how mitolineage effects on mitochondrial activity are manifested (*mitolineage×sex, mitolineage×study site, sex×study site, sex×season, season×study site, sex×mitolineage×season*). Up to 30 predictor variables could be included in each model (limitation of the program in the following step). When all variables could not be included, variables that were rarely selected in the previous step and/or high correlation with other selected variables were excluded. All numeric variables in this step were scaled and centred, because they spanned five orders of magnitude.

Modelling and model selection were automated using the *dredge* function of the R-package *MuMIn* (Bartoń 2018). This function uses a specified global model to assess all possible combinations of fixed terms within that model. We limited the number of predictors from one to three for the flux control ratio models. For the mitochondrial respiration rates dataset, two to four predictor variables were allowed, however, protein concentration was included in all of these models to correct for technical differences in blood cell quantity between samples. We ran four independent repetitions of dredging, and ranked models based on their average AICc scores. We specified a global model using *MCMCglmm* (Hadfield 2010), a Bayesian approach to multivariate generalized mixed models. By using multivariate models, we were able to assess whether and how predictor variables affect response variables, as well as their relationships with other variables (covariance). We specified a weakly informative inverse-Wishart prior for variance structures, which we tested against a more informative prior with no change in model statistics, confirming that the chosen priors did not affect posterior means of the model parameters, and that the data are informative. We assumed Gaussian distribution of the response variables with identity link functions (despite the control ratio responses being confined to 0-1, their distributions were near normal). We specified a global intercept, but no random effects, and ran MCMCs to 1,000,000 iterations with a thinning of 1,000 and a burn-in of 250,000, based on empirical run optimization.

S4.3: Conclusion averaging

To derive conclusions concerning the relationships among mitochondrial function, climate, weather and individual characteristics, we summarized their effects across models. Given the

restrictions of a small dataset and relatively high collinearity of variables, we evaluated the effects of predictors by summarising them only for the best models (ΔAICc <10). We evaluated convergence and independence of samples, and extracted posterior means and confidence estimates for each term. For each term present in the selected models we then assessed and summarized significance, directionality, and effect size across the different models.

Tables

Table S1: Central location of capture sites, climatic variation at capture locations for maximum summer temperature and minimal winter temperature, and mitolineages of birds captured at each site and assessed for mitochondrial function.

Site Name	Location (latitude, longitude)	Mitolineage Present (samples)	Max. Summer Temp. (˚C)	Min. Winter Temp. (˚C)
Pure Cold	-37.35, 144.23	B (27 B, 1 unassigned)	22.64 – 23.30 (range: 0.66)	2.03 – 2.23 (range: 0.2)
Hybrid Cold	-37.06, 144.08	B with a minority of A (8 A, 32 B, 1 unassigned)	27.47 – 27.81 (range: 0.34)	3.11 – 3.43 (range: 0.32)
Hybrid Hot	-36.82, 144.21	A with a minority of B (29 A, 9 B, 3 unassigned)	27.4 – 28.02 (range: 0.62)	3.25 – 3.75 (range: 0.5)
Pure Hot	-37.01, 143.75	A (21 A, 1 unassigned)	27.79 – 28.29 (range: 0.5)	3.49 – 3.79 (range: 0.3)

Table S2: Climate variables included in the variable-selection process. Climate Variable Class - Climate parameters were classified into Rainfall, Temperature, Evaporation, Evapotranspiration, Solar Exposure; Month(s) Averaged – the variables were summarised monthly and annually; Averaged Years – the years data of the variable was summarised; Description – a brief description of each group of variables. All shown data were obtained from the Australian Bureau of Meteorology.

#	Climate Variable Name	Climate Variable Class	Period	Averaged Years	Description
1	DaysofRain_Annual_1961-1990	Rainfall	Annual	1961-1990	"Days of rain": the average number of days, for each month and annually, when rainfall totals exceeded 1mm.
2	DaysofRain_Apr_1961-1990	Rainfall	Apr	1961-1990	
3	DaysofRain_Aug_1961-1990	Rainfall	Aug	1961-1990	
4	DaysofRain_Dec_1961-1990	Rainfall	Dec	1961-1990	
5	DaysofRain_Feb_1961-1990	Rainfall	Feb	1961-1990	
6	DaysofRain_Jan_1961-1990	Rainfall	Jan	1961-1990	
7	DaysofRain_Jul_1961-1990	Rainfall	Jul	1961-1990	
8	DaysofRain_Jun_1961-1990	Rainfall	Jun	1961-1990	
9	DaysofRain_Mar_1961-1990	Rainfall	Mar	1961-1990	
10	DaysofRain_May_1961-1990	Rainfall	May	1961-1990	
11	DaysofRain_Nov_1961-1990	Rainfall	Nov	1961-1990	
12	DaysofRain_Oct_1961-1990	Rainfall	Oct	1961-1990	
13	DaysofRain_Sep_1961-1990	Rainfall	Sep	1961-1990	
14	RainMeanAnnualVic_1961-1990	Rainfall	Annual	1961-1990	"Rain Mean": rainfall (in mm), averaged for each month and annually
15	RainMeanAprVic_1961-1990	Rainfall	Apr	1961-1990	
16	RainMeanAugVic_1961-1990	Rainfall	Aug	1961-1990	
17	RainMeanDecVic_1961-1990	Rainfall	Dec	1961-1990	
18	RainMeanFebVic_1961-1990	Rainfall	Feb	1961-1990	
19	RainMeanJanVic_1961-1990	Rainfall	Jan	1961-1990	
20	RainMeanJulVic_1961-1990	Rainfall	Jul	1961-1990	
21	RainMeanJunVic_1961-1990	Rainfall	Jun	1961-1990	
22	RainMeanMarVic_1961-1990	Rainfall	Mar	1961-1990	
23	RainMeanMayVic_1961-1990	Rainfall	May	1961-1990	
24	RainMeanNovVic_1961-1990	Rainfall	Nov	1961-1990	
25	RainMeanOctVic_1961-1990	Rainfall	Oct	1961-1990	
26	RainMeanSepVic_1961-1990	Rainfall	Sep	1961-1990	
27	RainVar_Annual_1976-2005	Rainfall	Annual	1976-2005	"Rain Var": Averaged variability in rainfall between months and years. Calculated by
28	RainVar_Apr.Jun_1976-2005	Rainfall	Apr.Jun	1976-2005	
29	RainVar_Aug.Oct_1976-2005	Rainfall	Aug.Oct	1976-2005	
30	RainVar_Dec.Feb_1976-2005	Rainfall	Dec.Feb	1976-2005	
31	RainVar_Feb.Apr_1976-2005	Rainfall	Feb.Apr	1976-2005	
32	RainVar_Jan.Mar_1976-2005	Rainfall	Jan.Mar	1976-2005	
33	RainVar_Jul.Sep_1976-2005	Rainfall	Jul.Sep	1976-2005	

#	Climate Variable Name	Climate Variable Class	Period	Averaged Years	Description
34	RainVar_Jun.Aug_1976-2005	Rainfall	Jun.Aug	1976-2005	subtraction of the 10_{th} percentile from the 90_{th} percentile, divided by the 50_{th} percentile.
35	RainVar_Mar.May_1976-2005	Rainfall	Mar.May	1976-2005	
36	RainVar_May.Jul_1976-2005	Rainfall	May.Jul	1976-2005	
37	RainVar_Nov.Jan_1976-2005	Rainfall	Nov.Jan	1976-2005	
38	RainVar_Oct.Dec_1976-2005	Rainfall	Oct.Dec	1976-2005	
39	RainVar_Sep.Nov_1976-2005	Rainfall	Sep.Nov	1976-2005	
40	Evap_Annual_1976-2005	Evaporation	Annual	1976-2005	"Evap": Evaporation of from an open pan (in mm), averaged monthly and annually.
41	Evap_Apr_1976-2005	Evaporation	Apr	1976-2005	
42	Evap_Aug_1976-2005	Evaporation	Aug	1976-2005	
43	Evap_Dec_1976-2005	Evaporation	Dec	1976-2005	
44	Evap_Feb_1976-2005	Evaporation	Feb	1976-2005	
45	Evap_Jan_1976-2005	Evaporation	Jan	1976-2005	
46	Evap_Jul_1976-2005	Evaporation	Jul	1976-2005	
47	Evap_Jun_1976-2005	Evaporation	Jun	1976-2005	
48	Evap_Mar_1976-2005	Evaporation	Mar	1976-2005	
49	Evap_May_1976-2005	Evaporation	May	1976-2005	
50	Evap_Nov_1976-2005	Evaporation	Nov	1976-2005	
51	Evap_Oct_1976-2005	Evaporation	Oct	1976-2005	
52	Evap_Sep_1976-2005	Evaporation	Sep	1976-2005	
53	EvapTrans_Annual_1961-1990	Evapotranspiration	Annual	1961-1990	"EvapTrans": Monthly and annually averaged evapotranspiration of from an open pan (in mm).
54	EvapTrans_Apr_1961-1990	Evapotranspiration	Apr	1961-1990	
55	EvapTrans_Aug_1961-1990	Evapotranspiration	Aug	1961-1990	
56	EvapTrans_Dec_1961-1990	Evapotranspiration	Dec	1961-1990	
57	EvapTrans_Feb_1961-1990	Evapotranspiration	Feb	1961-1990	
58	EvapTrans_Jan_1961-1990	Evapotranspiration	Jan	1961-1990	
59	EvapTrans_Jul_1961-1990	Evapotranspiration	Jul	1961-1990	
60	EvapTrans_Jun_1961-1990	Evapotranspiration	Jun	1961-1990	
61	EvapTrans_Mar_1961-1990	Evapotranspiration	Mar	1961-1990	
62	EvapTrans_May_1961-1990	Evapotranspiration	May	1961-1990	
63	EvapTrans_Nov_1961-1990	Evapotranspiration	Nov	1961-1990	
64	EvapTrans_Oct_1961-1990	Evapotranspiration	Oct	1961-1990	
65	EvapTrans_Sep_1961-1990	Evapotranspiration	Sep	1961-1990	
66	MaxTemp_Annual_1961-1990	Temperature	Annual	1961-1990	"Max Temp": Average annual and average monthly maximum
67	MaxTemp_Apr_1961-1990	Temperature	Apr	1961-1990	
68	MaxTemp_Aug_1961-1990	Temperature	Aug	1961-1990	
69	MaxTemp_Dec_1961-1990	Temperature	Dec	1961-1990	
70	MaxTemp_Feb_1961-1990	Temperature	Feb	1961-1990	
71	MaxTemp_Jan_1961-1990	Temperature	Jan	1961-1990	

#	Climate Variable Name	Climate Variable Class	Period	Averaged Years	Description
72	MaxTemp_Jul_1961-1990	Temperature	Jul	1961-1990	temperature
73	MaxTemp_Jun_1961-1990	Temperature	Jun	1961-1990	.
74	MaxTemp_Mar_1961-1990	Temperature	Mar	1961-1990	
75	MaxTemp_May_1961-1990	Temperature	May	1961-1990	
76	MaxTemp_Nov_1961-1990	Temperature	Nov	1961-1990	
77	MaxTemp_Oct_1961-1990	Temperature	Oct	1961-1990	
78	MaxTemp_Sep_1961-1990	Temperature	Sep	1961-1990	
79	MeanTemp_Annual_1961-1990	Temperature	Annual	1961-1990	
80	MeanTemp_Apr_1961-1990	Temperature	Apr	1961-1990	
81	MeanTemp_Aug_1961-1990	Temperature	Aug	1961-1990	
82	MeanTemp_Dec_1961-1990	Temperature	Dec	1961-1990	"Mean Temp": Average annual and average monthly average temperature
83	MeanTemp_Feb_1961-1990	Temperature	Feb	1961-1990	
84	MeanTemp_Jan_1961-1990	Temperature	Jan	1961-1990	
85	MeanTemp_Jul_1961-1990	Temperature	Jul	1961-1990	
86	MeanTemp_Jun_1961-1990	Temperature	Jun	1961-1990	
87	MeanTemp_Mar_1961-1990	Temperature	Mar	1961-1990	
88	MeanTemp_May_1961-1990	Temperature	May	1961-1990	
89	MeanTemp_Nov_1961-1990	Temperature	Nov	1961-1990	
90	MeanTemp_Oct_1961-1990	Temperature	Oct	1961-1990	
91	MeanTemp_Sep_1961-1990	Temperature	Sep	1961-1990	
92	MinTemp_Annual_1961-1990	Temperature	Annual	1961-1990	
93	MinTemp_Apr_1961-1990	Temperature	Apr	1961-1990	
94	MinTemp_Aug_1961-1990	Temperature	Aug	1961-1990	
95	MinTemp_Dec_1961-1990	Temperature	Dec	1961-1990	"Min Temp": Average annual and average monthly minimum temperature
96	MinTemp_Feb_1961-1990	Temperature	Feb	1961-1990	
97	MinTemp_Jan_1961-1990	Temperature	Jan	1961-1990	
98	MinTemp_Jul_1961-1990	Temperature	Jul	1961-1990	
99	MinTemp_Jun_1961-1990	Temperature	Jun	1961-1990	
100	MinTemp_Mar_1961-1990	Temperature	Mar	1961-1990	
101	MinTemp_May_1961-1990	Temperature	May	1961-1990	
102	MinTemp_Nov_1961-1990	Temperature	Nov	1961-1990	
103	MinTemp_Oct_1961-1990	Temperature	Oct	1961-1990	
104	MinTemp_Sep_1961-1990	Temperature	Sep	1961-1990	
105	RelHum15_Annual_1976-2005	Relative Humidity 3pm	Annual	1976-2005	"Rel Hum": Average annual and average monthly relative humidity at
106	RelHum15_Apr_1976-2005	Relative Humidity 3pm	Apr	1976-2005	
107	RelHum15_Aug_1976-2005	Relative Humidity 3pm	Aug	1976-2005	
108	RelHum15_Dec_1976-2005	Relative Humidity 3pm	Dec	1976-2005	
109	RelHum15_Feb_1976-2005	Relative Humidity 3pm	Feb	1976-2005	
110	RelHum15_Jan_1976-2005	Relative Humidity 3pm	Jan	1976-2005	

#	Climate Variable Name	Climate Variable Class	Period	Averaged Years	Description
111	RelHum15_Jul_1976-2005	Relative Humidity 3pm	Jul	1976-2005	3 pm. Relative to maximum moisture content of air.
112	RelHum15_Jun_1976-2005	Relative Humidity 3pm	Jun	1976-2005	
113	RelHum15_Mar_1976-2005	Relative Humidity 3pm	Mar	1976-2005	
114	RelHum15_May_1976-2005	Relative Humidity 3pm	May	1976-2005	
115	RelHum15_Nov_1976-2005	Relative Humidity 3pm	Nov	1976-2005	
116	RelHum15_Oct_1976-2005	Relative Humidity 3pm	Oct	1976-2005	
117	RelHum15_Sep_1976-2005	Relative Humidity 3pm	Sep	1976-2005	
118	RelHum9_Annual_1976-2005	Relative Humidity 9am	Annual	1976-2005	
119	RelHum9_Apr_1976-2005	Relative Humidity 9am	Apr	1976-2005	"Rel Hum": Average annual and average monthly relative humidity at 9am. Relative to maximum moisture content of air.
120	RelHum9_Aug_1976-2005	Relative Humidity 9am	Aug	1976-2005	
121	RelHum9_Dec_1976-2005	Relative Humidity 9am	Dec	1976-2005	
122	RelHum9_Feb_1976-2005	Relative Humidity 9am	Feb	1976-2005	
123	RelHum9_Jan_1976-2005	Relative Humidity 9am	Jan	1976-2005	
124	RelHum9_Jul_1976-2005	Relative Humidity 9am	Jul	1976-2005	
125	RelHum9_Jun_1976-2005	Relative Humidity 9am	Jun	1976-2005	
126	RelHum9_Mar_1976-2005	Relative Humidity 9am	Mar	1976-2005	
127	RelHum9_May_1976-2005	Relative Humidity 9am	May	1976-2005	
128	RelHum9_Nov_1976-2005	Relative Humidity 9am	Nov	1976-2005	
129	RelHum9_Oct_1976-2005	Relative Humidity 9am	Oct	1976-2005	
130	RelHum9_Sep_1976-2005	Relative Humidity 9am	Sep	1976-2005	
131	SolarEx_Annual_1990-2011	Solar Exposure	Annual	1990-2011	"Solar Ex": Average daily global solar exposure over Australia for each month, and annually. The daily global solar exposure (falling on a horizontal surface) are typically between 1 and 35 MJ/m_2
132	SolarEx_Apr_1990-2011	Solar Exposure	Apr	1990-2011	
133	SolarEx_Aug_1990-2011	Solar Exposure	Aug	1990-2011	
134	SolarEx_Dec_1990-2011	Solar Exposure	Dec	1990-2011	
135	SolarEx_Feb_1990-2011	Solar Exposure	Feb	1990-2011	
136	SolarEx_Jan_1990-2011	Solar Exposure	Jan	1990-2011	
137	SolarEx_Jul_1990-2011	Solar Exposure	Jul	1990-2011	
138	SolarEx_Jun_1990-2011	Solar Exposure	Jun	1990-2011	
139	SolarEx_Mar_1990-2011	Solar Exposure	Mar	1990-2011	
140	SolarEx_May_1990-2011	Solar Exposure	May	1990-2011	
141	SolarEx_Nov_1990-2011	Solar Exposure	Nov	1990-2011	
142	SolarEx_Oct_1990-2011	Solar Exposure	Oct	1990-2011	
143	SolarEx_Sep_1990-2011	Solar Exposure	Sep	1990-2011	

Table S3: Weather variables included for selection in variables selection process. Class – Weather variables were classified into are classed into Temperature, Rainfall, Vapour pressure, and Solar Exposure. Days – the number of days prior to the capture date a variable was summarised for. Description – a brief description of the variable set summarised. All shown data were obtained from the Australian Bureau of Meteorology.

#	Variable Name	Class	Days	Description
1	MaxT_Max03D	Temperature	3	The highest maximum temperature recorded in the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily maximum temperatures.
2	MaxT_Max07D	Temperature	7	
3	MaxT_Max14D	Temperature	14	
4	MaxT_Max21D	Temperature	21	
5	MaxT_Max28D	Temperature	28	
6	MaxT_Max56D	Temperature	56	
7	MaxT_Min03D	Temperature	3	The lowest maximum temperature recorded in the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily maximum temperatures.
8	MaxT_Min07D	Temperature	7	
9	MaxT_Min14D	Temperature	14	
10	MaxT_Min21D	Temperature	21	
11	MaxT_Min28D	Temperature	28	
12	MaxT_Min56D	Temperature	56	
13	MaxT_Mean03D	Temperature	3	The average maximum temperature recorded in the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily maximum temperatures.
14	MaxT_Mean07D	Temperature	7	
15	MaxT_Mean14D	Temperature	14	
16	MaxT_Mean21D	Temperature	21	
17	MaxT_Mean28D	Temperature	28	
18	MaxT_Mean56D	Temperature	56	
19	MinT_Min03D	Temperature	3	The lowest minimum temperature recorded in the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily minimum temperatures.
20	MinT_Min07D	Temperature	7	
21	MinT_Min14D	Temperature	14	
22	MinT_Min21D	Temperature	21	
23	MinT_Min28D	Temperature	28	
24	MinT_Min56D	Temperature	56	
25	MinT_Mean03D	Temperature	3	The average minimum temperature recorded in the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily minimum temperatures.
26	MinT_Mean07D	Temperature	7	
27	MinT_Mean14D	Temperature	14	
28	MinT_Mean21D	Temperature	21	
29	MinT_Mean28D	Temperature	28	
30	MinT_Mean56D	Temperature	56	
31	MinT_Max03D	Temperature	3	

#	Variable Name	Class	Days	Description
32	MinT_Max07D	Temperature	7	The highest minimum temperature recorded in the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily minimum temperatures.
33	MinT_Max14D	Temperature	14	
34	MinT_Max21D	Temperature	21	
35	MinT_Max28D	Temperature	28	
36	MinT_Max56D	Temperature	56	
37	TempRange_03D	Temperature	3	The difference between the highest maximum temperature and the lowest minimum temperature, as extracted for the individual's capture date and coordinates from the respective BOM provided gridded maps, in the days prior to capture
38	TempRange_07D	Temperature	7	
39	TempRange_14D	Temperature	14	
40	TempRange_21D	Temperature	21	
41	TempRange_28D	Temperature	28	
42	TempRange_56D	Temperature	56	
43	Rain_Max03D	Rainfall	3	The maximum rainfall (mm) in a single day recorded in the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily total rainfall.
44	Rain_Max07D	Rainfall	7	
45	Rain_Max14D	Rainfall	14	
46	Rain_Max21D	Rainfall	21	
47	Rain_Max28D	Rainfall	28	
48	Rain_Max56D	Rainfall	56	
49	Rain_Min03D	Rainfall	3	The minimum rainfall (mm) in a single day recorded in the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily total rainfall.
50	Rain_Min07D	Rainfall	7	
51	Rain_Min14D	Rainfall	14	
52	Rain_Min21D	Rainfall	21	
53	Rain_Min28D	Rainfall	28	
54	Rain_Min56D	Rainfall	56	
55	Rain_Mean03D	Rainfall	3	The average rainfall (mm) across the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily total rainfall.
56	Rain_Mean07D	Rainfall	7	
57	Rain_Mean14D	Rainfall	14	
58	Rain_Mean21D	Rainfall	21	
59	Rain_Mean28D	Rainfall	28	
60	Rain_Mean56D	Rainfall	56	
61	Rain_Sum03D	Rainfall	3	The sum of rainfall (mm) across the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily total rainfall.
62	Rain_Sum07D	Rainfall	7	
63	Rain_Sum14D	Rainfall	14	
64	Rain_Sum21D	Rainfall	21	
65	Rain_Sum28D	Rainfall	28	
66	Rain_Sum56D	Rainfall	56	
67	RainDaysZero_03D	Rainfall	3	

#	Variable Name	Class	Days	Description
68	RainDaysZero_07D	Rainfall	7	The number of days without any rainfall (0mm) during the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily total rainfall.
69	RainDaysZero_14D	Rainfall	14	
70	RainDaysZero_21D	Rainfall	21	
71	RainDaysZero_28D	Rainfall	28	
72	RainDaysZero_56D	Rainfall	56	
73	VapP09_Max03D	Vapour Pressure	3	The maximum vapour pressure recorded in the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily vapour pressure at 9 am.
74	VapP09_Max07D	Vapour Pressure	7	
75	VapP09_Max14D	Vapour Pressure	14	
76	VapP09_Max21D	Vapour Pressure	21	
77	VapP09_Max28D	Vapour Pressure	28	
78	VapP09_Max56D	Vapour Pressure	56	
79	VapP09_Min03D	Vapour Pressure	3	The minimum vapour pressure recorded in the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily vapour pressure at 9 am.
80	VapP09_Min07D	Vapour Pressure	7	
81	VapP09_Min14D	Vapour Pressure	14	
82	VapP09_Min21D	Vapour Pressure	21	
83	VapP09_Min28D	Vapour Pressure	28	
84	VapP09_Min56D	Vapour Pressure	56	
85	VapP09_Mean03D	Vapour Pressure	3	The average vapour pressure recorded in the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily vapour pressure at 9 am.
86	VapP09_Mean07D	Vapour Pressure	7	
87	VapP09_Mean14D	Vapour Pressure	14	
88	VapP09_Mean21D	Vapour Pressure	21	
89	VapP09_Mean28D	Vapour Pressure	28	
90	VapP09_Mean56D	Vapour Pressure	56	
91	VapP15_Max03D	Vapour Pressure	3	The maximum vapour pressure recorded in the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily vapour pressure at 3 pm.
92	VapP15_Max07D	Vapour Pressure	7	
93	VapP15_Max14D	Vapour Pressure	14	
94	VapP15_Max21D	Vapour Pressure	21	
95	VapP15_Max28D	Vapour Pressure	28	
96	VapP15_Max56D	Vapour Pressure	56	
97	VapP15_Min03D	Vapour Pressure	3	The minimum vapour pressure recorded in the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily Vapour Pressure at 3 pm.
98	VapP15_Min07D	Vapour Pressure	7	
99	VapP15_Min14D	Vapour Pressure	14	
100	VapP15_Min21D	Vapour Pressure	21	
101	VapP15_Min28D	Vapour Pressure	28	
102	VapP15_Min56D	Vapour Pressure	56	
103	VapP15_Mean03D	Vapour Pressure	3	

#	Variable Name	Class	Days	Description
104	VapP15_Mean07D	Vapour Pressure	7	The average vapour pressure recorded in the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily vapour pressure at 3 pm.
105	VapP15_Mean14D	Vapour Pressure	14	
106	VapP15_Mean21D	Vapour Pressure	21	
107	VapP15_Mean28D	Vapour Pressure	28	
108	VapP15_Mean56D	Vapour Pressure	56	
109	SolarExp_Max03D	Solar Exposure	3	The maximum solar exposure recorded in the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily solar exposure.
110	SolarExp_Max07D	Solar Exposure	7	
111	SolarExp_Max14D	Solar Exposure	14	
112	SolarExp_Max21D	Solar Exposure	21	
113	SolarExp_Max28D	Solar Exposure	28	
114	SolarExp_Max56D	Solar Exposure	56	
115	SolarExp_Min03D	Solar Exposure	3	The minimum solar exposure recorded in the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily solar exposure.
116	SolarExp_Min07D	Solar Exposure	7	
117	SolarExp_Min14D	Solar Exposure	14	
118	SolarExp_Min21D	Solar Exposure	21	
119	SolarExp_Min28D	Solar Exposure	28	
120	SolarExp_Min56D	Solar Exposure	56	
121	SolarExp_Mean03D	Solar Exposure	3	The mean solar exposure recorded in the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily solar exposure.
122	SolarExp_Mean07D	Solar Exposure	7	
123	SolarExp_Mean14D	Solar Exposure	14	
124	SolarExp_Mean21D	Solar Exposure	21	
125	SolarExp_Mean28D	Solar Exposure	28	
126	SolarExp_Mean56D	Solar Exposure	56	
127	SolarExp_Sum03D	Solar Exposure	3	The sum of the recorded solar exposure across the days prior to capture, as extracted for the individual's capture coordinates and capture date from the BOM provided gridded maps for daily solar exposure.
128	SolarExp_Sum07D	Solar Exposure	7	
129	SolarExp_Sum14D	Solar Exposure	14	
130	SolarExp_Sum21D	Solar Exposure	21	
131	SolarExp_Sum28D	Solar Exposure	28	
132	SolarExp_Sum56D	Solar Exposure	56	

Table S4: Individual variables included for selection in variables reduction process. Class – indicates if the variable was used as a continuous or a categorical predictor. Variable description – brief description of the variable. More details of additional body condition measurements (6 – 14) can be found in Box S1.

#	Variable name	Class	Variable description
1	Head-bill length	continuous	length measured in mm, to 0.1mm
2	Bill depth	continuous	depth measured in mm, to 0.1mm
3	Tarsus length	continuous	length measured in mm, to 0.1mm
4	Wing length	continuous	length measured in mm, to 1mm
5	Tail length	continuous	length measured in mm, to 1mm
6	Age	categorical	Adult, Sub-Adult, unassigned
7	Mass	continuous	body weight in g, to 0.5 g
8	Residual Condition (Tarsus)	continuous	Mass divided by tarsus length (Box S1)
9	Residual Condition (Head-Bill)	continuous	Mass divided by head-bill length
10	ScaledMass (Tarsus)	continuous	Mass corrected by head-bill length (Peig et al. 2009)
11	ScaledMass (Head_Bill)	continuous	Mass corrected by tarsus length (Peig et al. 2009)
12	Predicted Mass	continuous	Mass predicted by Size Score (linear regression)
13	Residual Mass	continuous	Residuals of linear regression of Mass ~ Size Score
14	Size Score	continuous	Additive score for head-bill, tarsus, wing, tail and bill lengths (scored were five inter-quantile ranges)
15	Sex	categorical	Male, Female, unassigned
16	Mitolineage	categorical	A, B, unassigned
17	Date	categorical	Date of capture, (38 different dates)
18	Trip ID	categorical	8 trips (1 - 8)
19	Month	categorical	7 months (2, 3, 6, 8, 9, 10, 11)
20	Season	categorical	Breeding, Summer, Winter
21	Study Site	categorical	Pure Cold, Shared Cold, Shared Hot, Pure Hot

Table S5: Summary of significant terms (predictor variables) in the best multivariate models (ΔAICc <10) for mitochondrial function measured as rates and flux control ratios across large and small spatial scales. Predictors were also classified into the three classes outlined in Tables S2-4. Each predictor variable is linked to a specific trait of mitochondrial respiration, and the corresponding parameter estimate (Avg. Post. Mean) is given with its upper and lower confidence interval (Avg. lCI, Avg. uCI). Models were replicated four-times and estimates averaged. The frequency of the significant predictor-response combination (Freq) for the shortlisted models for each of the the two datasets (Scale – Response) is also provided.

Predictor	Class	Response	Avg. Post. Mean	Avg. lCI	Avg. uCI	Freq	Scale - Responses
Bill	Individ.	CI/CI+CII	0.23	0.01	0.45	0.06	Small - Ratios
Evap_Apr_Histor.	Climate	L/CI	-0.25	-0.44	-0.07	0.02	Large - Ratios
Evap_Apr_Histor.	Climate	L/CI+CII	-0.30	-0.48	-0.10	0.02	Large - Ratios
Evap_Aug_Histor.	Climate	L/CI	-0.19	-0.38	-0.02	0.01	Large - Ratios
EvapTrans_Jul_Histor.	Climate	CI/CI+CII	0.19	0.00	0.36	0.01	Large - Ratios
EvapTrans_Jul_Histor.	Climate	L/CI	0.26	0.09	0.44	0.09	Large - Ratios
EvapTrans_Jul_Histor.	Climate	L/CI+CII	0.28	0.11	0.46	0.09	Large - Ratios
EvapTrans_Jun_Histor.	Climate	L/CI	0.22	0.04	0.38	0.10	Large - Ratios
EvapTrans_Jun_Histor.	Climate	L/CI+CII	0.23	0.06	0.40	0.13	Large - Ratios
EvapTrans_May_Histor.	Climate	CI/CI+CII	0.18	0.01	0.35	0.12	Large - Ratios
EvapTrans_May_Histor.	Climate	L/CI	0.24	0.07	0.41	0.35	Large - Ratios
EvapTrans_May_Histor.	Climate	L/CI+CII	0.29	0.11	0.47	0.40	Large - Ratios
MaxT_Max03D	Weather	CI/CI+CII	0.45	0.04	0.85	0.03	Small - Ratios
Protein concentration		CI	0.77	0.64	0.90	1.00	Large - Rates
Protein concentration		L	0.66	0.51	0.81	1.00	Large - Rates
Protein concentration		CI+CII	0.71	0.57	0.85	1.00	Large - Rates
Protein concentration		R	0.67	0.53	0.81	1.00	Large - Rates
Protein concentration		CI	0.70	0.54	0.86	1.00	Small - Rates
Protein concentration		L	0.60	0.42	0.78	1.00	Small - Rates
Protein concentration		CI+CII	0.64	0.46	0.81	1.00	Small - Rates
Protein concentration		R	0.73	0.57	0.89	1.00	Small - Rates
MinT_Min07D	Weather	CI/CI+CII	0.24	0.06	0.42	0.03	Large - Ratios
MinT_Min07D	Weather	L/CI	0.25	0.06	0.44	0.02	Large - Ratios
MinT_Min07D	Weather	L/CI+CII	0.23	0.05	0.40	0.07	Large - Ratios
RainDaysZero_07D	Weather	CI	0.27	0.11	0.42	0.25	Small - Rates
RainDaysZero_07D	Weather	L	0.28	0.11	0.46	0.25	Small - Rates
RainDaysZero_07D	Weather	CI+CII	0.21	0.04	0.38	0.25	Small - Rates
RelHum15_Mar_Histor.	Climate	L/CI+CII	0.20	0.02	0.39	0.02	Large - Ratios

Predictor	Class	Response	Avg. Post. Mean	Avg. lCI	Avg. uCI	Freq	Scale - Responses
RelHum15_Sep_Histor.	Climate	L/CI	0.22	0.04	0.40	0.02	Large - Ratios
RelHum15_Sep_Histor.	Climate	L/CI+CII	0.27	0.10	0.44	0.02	Large - Ratios
RelHum9_May_Histor.	Climate	L/CI	0.25	0.02	0.47	0.13	Small - Ratios
TempRange_03D	Weather	CI/CI+CII	0.29	0.05	0.53	0.11	Small - Ratios
VapP09_Min07D	Weather	CI/CI+CII	0.21	0.03	0.39	0.15	Large - Ratios
VapP09_Min07D	Weather	L/CI	0.27	0.05	0.48	0.21	Large - Ratios
VapP09_Min07D	Weather	L/CI+CII	0.30	0.10	0.51	0.23	Large - Ratios
VapP15_Mean14D	Weather	L	0.20	0.02	0.39	0.25	Large - Rates
VapP15_Mean56D	Weather	CI/CI+CII	-0.21	-0.39	-0.02	0.02	Large - Ratios
VapP15_Mean56D	Weather	L/CI	-0.21	-0.40	-0.03	0.05	Large - Ratios
VapP15_Mean56D	Weather	L/CI+CII	-0.24	-0.42	-0.06	0.07	Large - Ratios
VapP15_Mean56D	Weather	CI	0.17	0.04	0.31	0.25	Large - Rates
VapP15_Mean56D	Weather	L	0.24	0.09	0.39	0.25	Large - Rates
VapP15_Mean56D	Weather	CI+CII	0.16	0.02	0.30	0.25	Large - Rates
VapP15_Mean56D	Weather	R	0.17	0.02	0.30	0.25	Large - Rates
VapP15_Mean56D	Weather	CI	0.28	0.12	0.43	0.25	Small - Rates
VapP15_Mean56D	Weather	L	0.33	0.13	0.54	0.50	Small - Rates
VapP15_Mean56D	Weather	CI+CII	0.26	0.06	0.47	0.50	Small - Rates
VapP15_Min56D	Weather	CI	-0.20	-0.35	-0.06	0.81	Large - Rates
VapP15_Min56D	Weather	L	-0.21	-0.36	-0.06	0.25	Large - Rates
VapP15_Min56D	Weather	CI+CII	-0.18	-0.33	-0.03	0.38	Large - Rates

CHAPTER 3

Linking mitochondrial genetic variation to metabolic rate and evaporative water loss in a passerine

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Abstract

Mitochondrial DNA (mtDNA) variation is thought to convey adaptation to climate in many organisms, but the underlying chain of function is not well understood. By encoding and regulating protein complexes responsible for oxidizing nutrients and making available cellular energy, mtDNA variation can affect mitochondrial function, resulting in different whole-body metabolic phenotypes. Adaptations to hot and arid environments have been linked to lower metabolic expenditure of resting individuals in thermoneutral conditions (basal metabolic rate), and lower evaporative water loss.

Here we investigated whether two mitochondrial lineages (mitolineages, mito-A and mito-B) of Eastern Yellow Robin (EYR), which exhibit a spatial distribution strongly associated with a gradient in maximum summer temperature, underlie metabolic phenotypes that might be adaptive to different climates. In testing this hypothesis, we account for seasonal changes in metabolic phenotype as well as plastic changes associated with recent weather conditions. We measured metabolic rate and evaporative water loss via open-flow respirometry in four wild populations, encompassing typical environments for each mitolineage (mito-A: hot, arid and variable vs. mito-B: cool, mesic and consistent), as well as sites where the two mitolineages share intermediate environments.

Regardless of their location, EYR of the mito-A lineage showed lower basal metabolic rate than did mito-B birds, but only during autumn. In contrast to this seasonal adjustment in mito-A birds, mito-B birds showed no significant adjustments of basal metabolic rate between the assessed seasons. Birds that experienced a wider range of temperature in the eight weeks before their assessments showed lower basal metabolic rates and shifted the temperature range in which they did not need to expend energy on thermoregulation (thermoneutral zone).

Evaporative water loss showed little association with mitolineage nor temperature range experienced prior to measurement, but decreased with a rise in the minimum humidity experienced and was higher at sites where conditions were in the coolest, more mesic and more consistent.

Our results suggest that seasonal differences in basal metabolic rates of birds of the mito-A lineage might reflect adaptation to hot and variable environments, but manipulative experiments under controlled conditions are needed to further test this possibility.

Keywords: [1] Bioenergetics, [2] Whole-organism respiration, [3] Eco-physiology, [4] Aves, [5] $\dot{V}CO_2$, [6] Climate adaptation

Introduction

The mechanisms through which mitochondrial DNA (mtDNA) variation affects whole-organism complex traits and contribute to climate adaptation are not well understood. The oxidative phosphorylation pathway is potentially an important mediator of the link between mtDNA variation and whole-organism complex traits (Pichaud *et al.* 2011, 2012; Wolff *et al.* 2016). MtDNA encodes some protein subunits of the complexes forming this pathway, and is also involved in its regulation (Rand *et al.* 2004; Gershoni *et al.* 2009). Because oxidative phosphorylation supports most of an organism's energetic needs by transforming energy from food metabolites to available cellular ATP, this pathway influences multiple levels of biological organization, but most directly whole-organism metabolic activity (Albertin *et al.* 2013; Toews *et al.* 2014a; Jin *et al.* 2018).

Metabolic rate and evaporative water loss are thought to contribute to an individual's adaptation to climate (Lillywhite 2006; White *et al.* 2007b; Burton *et al.* 2011). Differences in the temperature profile between environments have been identified as the main abiotic drivers of metabolic variation and likely adaptation in mammals and birds (Speakman and Rowland 1999; Tieleman and Williams 2000b; Lovegrove 2003; Rezende *et al.* 2004; White *et al.* 2007a; Jetz *et al.* 2012; Naya and Bozinovic 2012; Stager *et al.* 2016; Naya *et al.* 2018).

Most studies on endotherms focus on a sole measure of metabolism: basal metabolic rate (BMR), i.e. the rate of energy expenditure of resting individuals measured within the thermoneutral zone (TNZ). The TNZ is the range of ambient temperatures at which the organism can support their body temperature without regulatory changes in metabolic heat production or active heat loss. BMR is measured in individuals that are awake but inactive and are not expending energy on reproduction or digestion. Generally, basal metabolic rate is higher for species that live in colder environments (Tieleman *et al.* 2003a; McKechnie 2008). It is thought that the endotherms from colder environments have a higher BMR to allow them to persist via an increase of metabolic heat production, while in hot environments metabolic heat production is minimized (Humphries *et al.* 2005; Naya *et al.* 2018).

Acute increases of metabolic rate of an individual at ambient temperatures above and below the thermoneutral zone are generally related to the maintenance of a constant body temperature (Scholander *et al.* 1950). An elevated metabolism below the lower critical temperature (Tlc, the lower bound of the TNZ) is suggested to be caused by an increase in metabolic heat production. Above the upper critical temperature (Tuc, the upper bound of the

TNZ) an elevated metabolism largely results from active dissipation of endogenous heat and mechanisms minimizing heat gain from the environment (Dawson and Schmidt-Nielsen 1966; Weathers and Caccamise 1975; Tieleman and Williams 1999). Individuals adapted to cooler climates are expected to have lower thermal conductance (rates of heat transfer from the body to the environment) such that metabolic rates can be minimally elevated at low temperatures, while their metabolism above the TNZ is likely to be relatively high due to the energetic cost of the active heat dissipation needed to counteract higher rates of endogenous heat production and lower thermal conductance (Williams 1996).

Evaporative water loss (EWL) is a key mechanism to dissipate excess heat in mammals, and the main one in birds (Lillywhite 2006; McKechnie and Wolf 2010; Smith *et al.* 2015). EWL increases steeply at ambient temperatures above the upper critical temperature. EWL in individuals adapted to hot climates follows two avenues; 1) EWL is high in environments where water is not limiting (i.e. in tropics), and 2) EWL is low in arid environments because less metabolic heat is produced and/or, less environmental heat is absorbed and/or an increase in body temperature is tolerated (Tieleman and Williams 2002; Smith *et al.* 2015; Gerson *et al.* 2019).

Studies relating differences in whole-body metabolism do not consistently show a link to mitochondrial function, especially in wild organisms (Norin and Metcalfe 2019). Here, we use the Eastern Yellow Robin (*Eopsaltria australis*, hereafter EYR), an Australian songbird, as model system for testing the link between mitochondrial DNA variation and metabolic phenotype. EYR harbours two climate-associated mitochondrial lineages (mitolineages, mito-A and mito-B) (Pavlova *et al.* 2013; Morales *et al.* 2017). Their mitochondrial genomes differ in 6.8% of nucleotides, which is a level of difference often seen between species of birds (Avise and Walker 1998). EYRs bearing these different mitolineages have largely non-overlapping geographic distributions, despite low nuclear genetic differentiation between them. At narrow contact zones between populations bearing the two mitolineages, hybridization occurs (Morales *et al.* 2017). In the southern part of EYR's distribution, mito-A EYR are found almost exclusively in inland, more arid and climatically variable areas with warmer summers, whereas mito-B birds occur in more temperate and climatically stable coastal areas. The geographic distributions of mitolineages are associated with maximum temperature of the warmest month and precipitation of the driest month (Pavlova *et al.* 2013; Morales *et al.* 2017). Five amino acid replacements in Complex I of the oxidative phosphorylation pathway fixed between the mitolineages were inferred to be under positive selection, and predicted to significantly change

physio-chemical properties of subunits in Complex I, impacting the functions of the complex and subsequently that of the oxidative phosphorylation pathway (Morales *et al.* 2015; Lamb *et al.* 2018). It was hypothesized that via the encoding and regulating processes of oxidative phosphorylation that translate to whole-body metabolism, mitolineages in EYR can convey metabolic adaptation (Sunnucks et al 2017). Accordingly, arid-adapted mito-A birds are predicted to have lower metabolism and EWL compared to mito-B birds adapted to cooler and more mesic climates.

Here, we test whether mitolineages predict metabolic rate and EWL in EYR. We also include weather and climate variables to account for phenotypic plasticity and climatic effects on metabolic rate uncorrelated to mitolineage membership, respectively. We focus on spatially-proximate sites with different climatic characteristics, and also test both mitolineages at intermediate shared locations to isolate mitolineage effects if present. We expect that birds of the with mito-A mitolineage to have 1) a lower BMR, 2) a TNZ shifted upwards to higher temperatures and, 3) reduced EWL when compared to their mito-B conspecifics. In shared sites we expect the differences between the mitolineages to persist, supporting that mitochondrial genetic variation conveys putatively climate-adapted metabolic differences.

Methods

Study Sites and Field Sampling

The study was conducted in South-Eastern Australia, between March 2017 and July 2018. To minimize disruption to the breeding success of individuals, respirometry experiments did not take place during the breeding season between spring and early summer (August – December). Consequently, measurements were taken in three seasons only: summer, autumn, and winter.

Four study sites were chosen to represent subtle differences in climate, represented by maximum summer and minimum winter temperatures. At two sites of contrasting climates, referred to as Pure-Hot and Pure-Cold (Figure 1), EYR of only a single mitolineage (mito-A and mito-B, respectively) were present. At the other two sites, referred to as Shared-Hot and Shared-Cold, EYR of both mitolineages were present, with documented cases of interbreeding between them (Walters, unpublished Honours thesis, Monash University). This design was to enable comparisons of the two mitolineages side-by-side in 'shared' sites, in addition to measuring metabolic parameters in each mitolineage's putatively adapted 'pure' environment.

EYR (n=154)[1] were caught with mist-nets or spring traps, colour-banded, weight (Pesola Spring Balance 50 g, precision = 0.5 g), measured (weight, head-bill length, bill depth, tarsus length, wing length, tail length), aged, and assessed for moult. A blood sample (<75 µl) was taken with non-heparinised microcapillaries from the brachial vein for genetic analyses. These samples were immediately transferred into 1 ml of absolute ethanol and stored at -20 °C after return from the field. Birds, captured within the last four hours before sunset, were taken to the laboratory where respirometry measurements took place overnight (see below). EYR were fasted for a minimum of two hours prior to respiration measurements (Secor 2009). Measurements were taken at least two hours after sunset and continued for up to eight hours. Some of these birds had a further blood sample for analysis of mitochondrial activity after completion of the respiration experiment (see Chapter IV). At sunrise, EYR were released at the site of capture.

[1] Studies investigating variation in whole-body metabolism across a wide range of experimental temperatures in birds commonly have small sample sizes (<10 per treatment), and do commonly not consider season as a source of variation. In our study we consider our sample size sufficient because we only have six treatment groups (four study sites and two mitolineages (only present at two of the four sites).

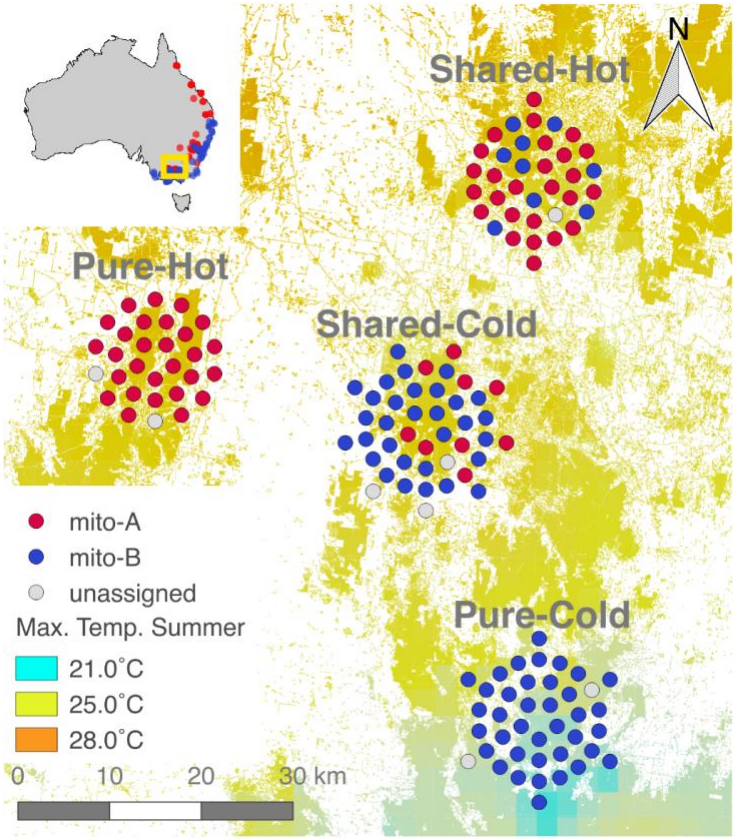

Figure 1: Spatial distribution of mitolineages (red dots- mito-A, blue dots- mito-B) across the four study sites in Central Victoria, Australia (dots representing individual birds are displaced for better visibility. Grey dots indicate individuals with unknown mitolineage). The background shading indicates woodland areas (EYR habitat) coloured according to the 30-year average maximum summer temperatures. White background indicates agricultural or urban non-habitat of EYR. The inset shows distribution of mitolineages throughout Australia and the approximate location of the study area (rectangle). Map created using QGIS (QGIS Development Team 2019).

Genetic Analyses

Total genomic DNA was extracted from whole blood using DNeasy Blood and Tissue extraction kit, QIAGEN, Germany, following manufacturer's instructions. The sex of each individual was assigned using sexing PCR, based on length variation in sex-linked CHD genes and mitolineage was assigned based on sequence variation in mtND2 gene (Genbank accession pending), following Pavlova *et al.* (2013).

Respirometry

We used standard positive-pressure open-flow multi-channel respirometry to measure oxygen consumption ($\dot{V}O_2$), carbon dioxide production ($\dot{V}CO_2$) and evaporative water loss of EYR at a range of ambient temperatures ($\dot{V}H_2O$), details below). While in the respirometry chambers,

EYR were exposed to temperatures ranging from 4.5 to 38.7 °C. At temperatures nearing ~31 °C and above EYRs displayed elongated posture, panting, and compressed plumage. At high experimental temperatures, some birds became unsettled and temperature was lowered until they returned to a calm state. Temperatures at the lower end of the measurement range triggered adjustments in plumage and posture opposing to the ones at hot temperatures. Respiration of each bird was measured at a minimum of three different temperatures (in random order) each night, randomly chosen such that each bird was measured at least once within each of three ranges; below (<20 °C), within (20-30 °C) and above (>31 °C) the anticipated thermoneutral zone based on (Khaliq *et al.* 2015). Covering a relevant range of temperatures that EYR experiences in the wild allowed us to infer population- and mitolineage-wide estimates of BMR, TNZ and thermal conductance.

Air was pumped (AT-520A compressor, Sparmax) from outside the facility and scrubbed of carbon dioxide (Soda Lime, #SL020, ChemSupply) and water vapour (Drierite, #26840, Hammond Drierite Co Ltd.), then split into eight separate air streams with a controlled airflow of 500 ml/min (GFC17 Mass-Flow Controllers, Aalborg), which was passed through eight respiratory chambers. Mass flow controllers were calibrated using a NIST-traceable bubble film flowmeter (1–10–500 ml; Bubble-O-Meter, Dublin, OH, USA). Respirometry chambers were clear cylindrical two-litre polypropylene (BPA free) airtight screw-top containers, with small air inlets and outlets. Natural posture of EYR was encouraged by small wooden perches at approximately 7 cm height mounted onto a plastic mesh on the bottom of the chamber. Temperature of the chambers was regulated using a custom-built temperature cabinet with a heater/cooler system (AC-073, TE Technology) and a temperature controller (TC-36-25-RS232, TE Technology). Temperature inside the chambers were recorded with iButtons (Maxim Thermochron DS1921G, accuracy: ±1.0°C)) or calibrated thermistors.

The excurrent airstreams were appropriately directed by a modified flow multiplexer (RM-8, SableSystems) through one of two CO_2/H_2O analysers (Li-840A, LI-COR) and a dual-channel O_2 analyser (Oxzilla II, Sable Systems). These instruments were interfaced with an ADInstruments PowerLab 16/35 A/D convertor, which also controlled the multiplexer and recorded the temperature measured by the thermal probes. Changes in air composition and variation between gas sensors were accounted for by measuring one control chamber (EYR absent) for each temperature, sensor and airstream.

EYR inside the chambers were constantly monitored via live visuals from cameras inside the thermo-regulated cabinet. If birds did not settle, they were removed from the respiratory chambers and excluded from experiments. Birds that did not maintain a still posture for extended periods were excluded from the analysis.

Mean rates (five-minute averages) of oxygen consumption, carbon dioxide production and evaporative water loss were extracted using LabChart (ADinstruments) for each temperature. Each measure was taken after an acclimation period to a new temperature of at least 40 minutes plus a 20-minute period where the bird's activity and respiration was actively monitored to ensure stable readings of an inactive bird. Rates of O_2 consumption ($\dot{V}O_2$), CO_2 production ($\dot{V}CO_2$), and water loss ($\dot{V}H_2O$) were calculated using standard equations (Lighton 2008, equations 9.7, 9.8 and 9.9, respectively). (Schmidt-Nielsen 1997).

Modelling Metabolic Rate and Evaporative water loss

Under the null hypothesis (H0), only climate and weather variation will predict metabolic variation (via long-term adaptation unrelated to mitolineage or plastic responses, respectively). We compare this null hypothesis with an alternative hypothesis (H1) that mitolineage predicts variation in metabolism, having accounted for differences among sites and seasons). We contrasted models that included mitolineage to models that summarise the null hypothesis. H0 models were further split into two groups, 1) models that contained variables that were identified as a predictor of mitochondrial function in EYR (Chapter 2), and 2) models with variables commonly linked to metabolic rate (Withers *et al.* 2006; Jetz *et al.* 2007; White *et al.* 2007a; Withers and Cooper 2014; Naya *et al.* 2018). Model quality comparisons were based on the Akaike Information Criterion (AIC).

Modelling metabolic rates estimated using $\dot{V}CO_2$

We used $\dot{V}CO_2$ rather than $\dot{V}O_2$as a measure of metabolic activity, because $\dot{V}CO_2$ had more stable readings. The median respiratory quotient (ratio of $\dot{V}CO_2$ to $\dot{V}O_2$) of all measurements (0.724) suggested that the individuals were in a post-absorptive stage (Walsberg and Wolf 1995). This showed that $\dot{V}CO_2$ and $\dot{V}O_2$ carried the same information (Figure A1).

Because whole-organism metabolic rate increases with mass, we corrected $\dot{V}CO_2$ for mass; not fitting mass as a variable enabled us to reduce the complexity of our models. The scaling exponent of mass was derived with linear mixed effect models in R (R Core Team 2018) and RStudio (RStudio Team 2016) using lme4 (Bates *et al.* 2015), in which we modelled the log10

of basal metabolic rate as a function of log10 of mass, as well as sex, mitolineage and study site. The best model (AIC criterion) with a significant estimate of log10 of mass was chosen and provided the scaling exponent (0.445). The respiratory rates were then adjusted with the scaling corrected mass ($\dot{V}CO_2$ $(mass_{0.445})_{-1}$).

To assess the effects of individual and environmental variation on metabolic rate we used nonlinear mixed effects models. The basic model fitted segmented models predicting metabolic rates, following the Scholander-Irving model with three parts, as a function of ambient temperature using the nlme package (Pinheiro *et al.* 2019). In the first segment, here referred to as Cmin segment, metabolic rate is a linear function of temperature below the lower critical temperature (Tlc) of the thermoneutral zone (TNZ); the slope (Cmin) of this line is an estimate of the minimal thermal conductance at different experimental temperatures (Figure 2). In the second segment of the model (here called the BMR segment), metabolic rate is independent of temperature and the elevation (=intercept) of this segment is an estimate of basal metabolic rate (BMR). The temperature at which the first and second segments intersect provides an estimate of Tlc. The third segment starts with Tuc and reflects linear increase in metabolic rate due to heat loss. For EYR, the data collected in this third segment, above the approximated upper critical temperature (~28 °C), were excluded from the analysis because there was no significant increase of metabolic rate with temperature identified by non-linear mixed effect models with three segments. Therefore, three parameters of this general model (Cmin, Tlc and BMB), describing first two segments of the Scholander-Irving model, were used to model a population-wide response. Additional explanatory variables were added to each model segment as outlined in detail below. Repeated measurements of the same EYR during at different temperatures required an individual identifier to be included as a random effect in our models. We tested all possible random effect structures on the basic model of $\dot{V}CO_2$ as a function of experimental temperature, and chose the one that yielded models of the highest quality (AIC criterion), resulting in a random effect on the BMR segment only.

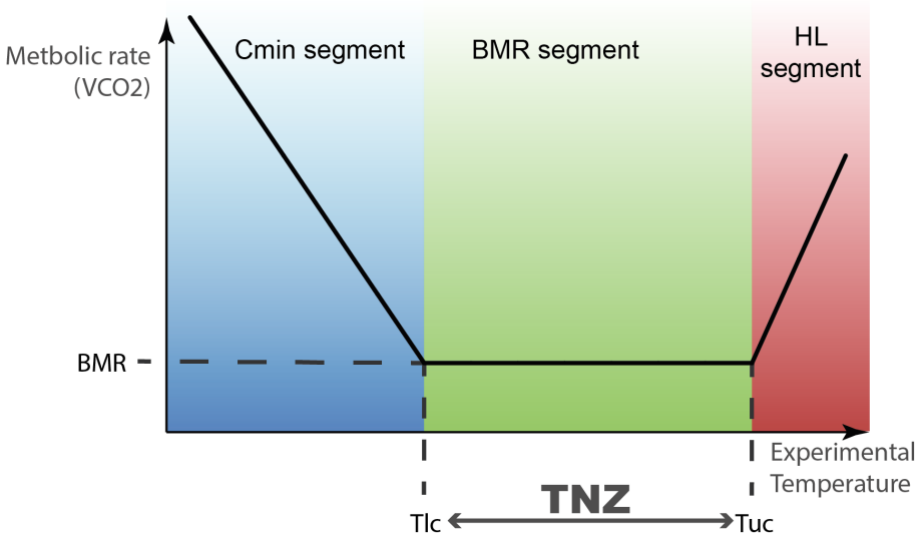

Figure 2: Graphical representation of the non-linear models built to describe mitochondrial function as a function of experimental temperature. The thermoneutral zone (TNZ) is defined by the lower and upper critical temperature (Tlc and Tuc, respectively), and defines the temperature range at which the BMR segment describes the relationship of metabolic rate and temperature). At temperatures below the critical lower temperature this relationship is described by the Cmin segment. At temperatures above the upper critical temperature the HL segment describes this relationship. Lower and upper critical temperatures themselves are described by the intersection of modelling segments.

To find the model that best explains variation in metabolic rate among H1 and H0 models, we compared models with different fixed effects. H1 and H0 models were built to explain variation in one of the three response variables (Cmin, Tlc and BMB). H1 models included from one to all of the following fixed effect terms: sex, mitolineage, study site and season and their possible interactions. H0 models based on Chapter 2 included one of the following 14 variables (eight climate and six weather variables): number of days without rain in the 7 days prior to capture, vapour pressure (56-day mean and minimum, 14-day mean and 7-day minimum), 7-day minimum temperature, 30-year averaged monthly evapotranspiration of May, June, and July, 30-year averaged monthly relative humidity of May, September, and March, and 30-year averaged monthly historical evaporation of August, and April. H0 models based on temperature variables commonly linked to metabolic rate included one of the following 12 variables (six climate and six weather variables): 30-year averaged maximum, mean and minimum temperature, 30-year averaged maximum temperature of the hottest month (February), 30-year averaged minimum temperature of the coldest month (July), 30-year averaged temperature range, and maximum, minimum and range of temperatures in the 7 and 56 days prior to capture.

Climate and weather data were sourced from the Australian Bureau of Meteorology (Commonwealth of Australia 2018b). Estimates of the variables were extracted for bird capture locations from grids (resolution of 5 x 5 km or higher) with the Geographic Data Analysis and Modelling package raster (Hijmans 2018).

To investigate whether the basal metabolic rates of birds of the two mitolineages differed in the shared environments, the best $\dot{V}CO_2$ model was simplified, as follows. The original metabolic measurements for birds from shared sites were subsetted based on the TNZ estimated from the best H1 model, to produce season-specific measurements of BMR. Linear models of BMR were tested including the same predictors as for the H1 models.

Modelling evaporative water loss measured using $\dot{V}H_2O$

Effects of individual and environmental variation on evaporative water loss were assessed by comparison of linear mixed effect models. As for $\dot{V}CO_2$ models, an individual identifier was implemented as a random effect and models were categorised into H0 and H1 models including the same predictors. We corrected for heteroskedasticity within the data, because variance necessarily increases with mean water loss (because water loss cannot be less than zero, thus low values will have a lower bound of zero that applies less to higher values). Different corrections were tested, and the one resulting in the best AIC score was implemented. Modelling and model comparison was automated using the dredge function (Bartoń 2018) and a global lme model (Pinheiro *et al.* 2019). All models fitted a second order polynomial and contained ambient temperature and mass as fixed terms. Dredging of H0 models was limited to include only one additional fixed term, whereas H1 models fit all possible combinations of main terms and interactions.

Results

To investigate whether the two mitolineages of EYR show climate adapted metabolic phenotypes, we compared models supporting the null hypothesis (climate/weather as drivers) with those supporting the alternative hypothesis (genetics/site-specific variation as drivers), and also included a comparison of merged models, combining the strongest drivers of each hypothesis. Considering only the best models, we found that individuals of mito-A lineage showed a lower mass-specific BMR in Autumn than did mito-B individuals. In contrast, mito-B birds showed little seasonal change. This was the only significant difference identified between the two mitolineages in the fitted $\dot{V}CO_2$ models. However, when considering mean mass-uncorrected rates of CO_2 production $\dot{V}CO_2$ within the thermoneutral zone across all seasons and study sites were 0.767 (SD=0.138) ml min_{-1}, with the mean for the mito-B mitolineage being significantly higher than that of mito-A by ~5% (t = -2.311, df = 222.92, p = 0.0218, Figure 3.A). The population wide mean of $\dot{V}H_2O$ was 1.999 (SD=0.783). Mito-B birds showed ~10% higher $\dot{V}H_2O$ in the thermoneutral zone compared to mito-A ones, but this difference was not significant (0.198, t = -1.775, df = 203.98, p = 0.077, Figure 3.B).

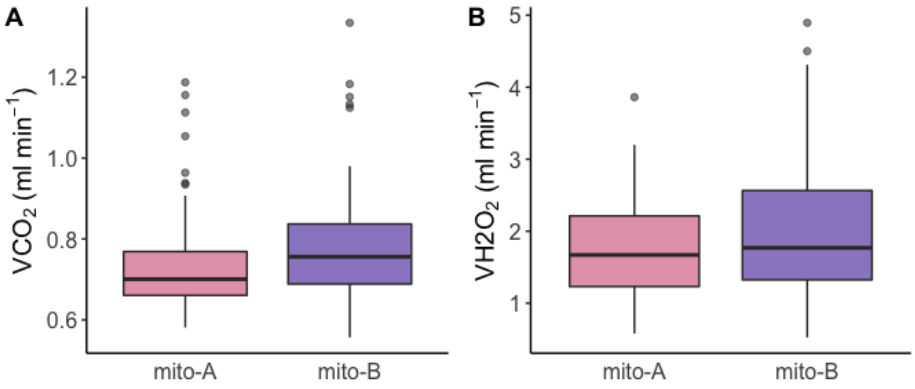

Figure 3: Distribution of basal metabolic rate estimates (A) and minimal evaporative water loss (B) for values falling into the estimated thermoneutral zone estimated in the best H1 model of metabolic rate (Table 2), across all seasons and sites.

Metabolic rate ($\dot{V}CO_2$) models

Variation in mass-corrected metabolic rate was explained equally well by H1 and H0 models ($\Delta AIC<2$). H0 models identified the maximum temperature and the range of temperatures experienced by EYR in the wild as well as humidity as good predictors of mass-corrected metabolic rate (Table A1). H1 models consistently showed the importance of seasonality,

mitolineage membership and their interaction (Table A2). There was no support for mass-corrected metabolic rate differing between the sexes.

The temperature range experienced by the EYR in the 56 days prior to capture was the best predictor of BMR and Tlc parameters of the mass-corrected metabolic rate model in the H0 models (AIC -1080.66): basal metabolic rate declined significantly by -0.002 for each degree increase in temperature range. Temperature range had a larger effect on the lower critical temperature, increasing it by 0.628 °C (± 0.126 SE) for every degree increase in temperature range (Table A3).

In the eight best H1 models, mitolineage, season and their interaction were the best predictors of parameters of mass-corrected metabolic rate (Table A2). The single best model (AIC -1081.11) identified a significant interactive effect of season × mitolineage on BMR, and season on Tlc, but no effect on Cmin (Table A4). Mito-A birds' BMR was 0.033 (± 0.008 SE) higher in winter than in autumn, while mito-B birds had similar BMR in all seasons. Tlc changed significantly with season, being lowest in winter and highest in summer.

The seasonal effects described above could be related to EYR life history events, such as moulting, or might reflect variation in weather according to season. To test whether the effect of season and/or mitolineage persisted in the H1 models if plastic responses to weather were controlled for, we added the best weather predictor (temperature range 56d) to the best model of H1 (effect on Tlc and BMR only). This new model was of better quality than any of the other H1 or H0 models (AIC -1088.52), and the relationships of season and mitolineage with BMR remained: mito-A individuals were still estimated to have a lower BMR, compared to mito-B ones, but only in autumn (Figure 4). However, inclusion of temperature range removed the effect of season on Tlc (Table 1). To isolate the effect of mitolineage in the shared sites, reduced models (containing only data from these shared sites) were built. These included only estimates of mass-corrected BMR, i.e. measurements that were recorded at experimental temperatures within the season specific TNZ (Table A4). In none of these models was mitolineage a significant predictor of BMR. Only season showed a significant effect, with BMR increasing in the winter (Table 1).

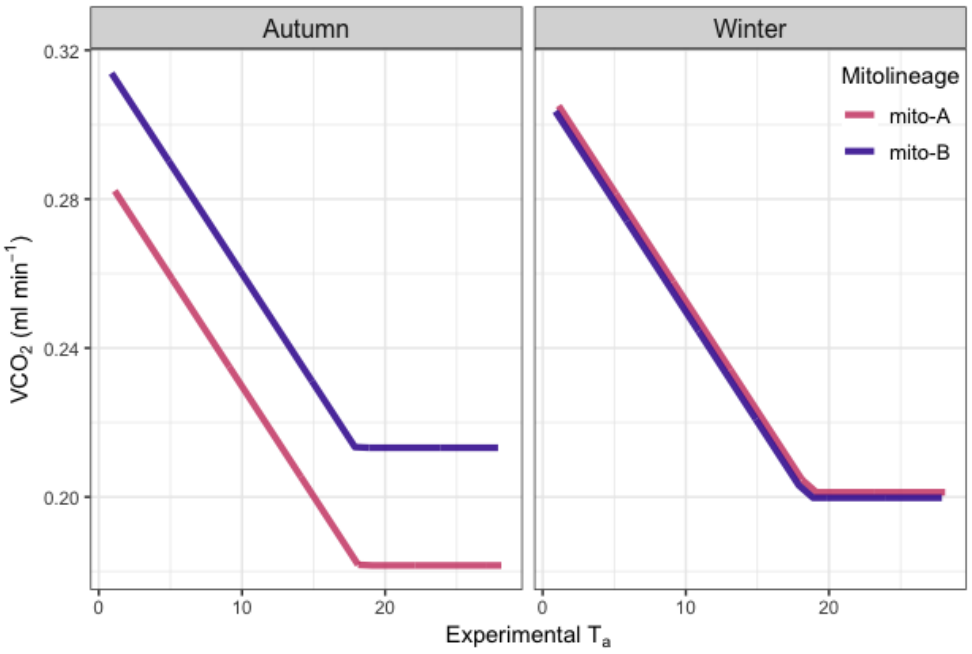

Figure 4: Effect of mitolineage and season on mass-corrected metabolic rate (VCO_2). BMR changes significantly from autumn to winter in birds of the mito-A lineage only. Cmin and Tlc is independent of mitolineage and season effects in this combined model (Table 1). Data includes all sites. Values were predicted based on the best H1-H0 combined model (Table 1). The term temperature range was fixed to its mean for each season. The estimates in winter were identical, but were slightly displaced for visibility.

Table 1: Parameter estimates for the model combining the best H1 and H0 model of metabolic rate (VCO_2). b1 indicates the intercept and b2 the slope of each linear compound model. A random effect of individual identity of EYR on BMR was included. The AIC for this model was -1088.52.

	Value	Std. Error	df	t-value	p-value
b1.BMR	0.250	0.022	175	11.520	0.000
b1.Cmin	0.006	0.001	175	10.093	0.000
b1.Tlc	2.243	4.471	175	0.502	0.617
b2.BMR.Summer	0.0190	0.017	175	1.135	0.258
b2.BMR.Autumn	-0.022	0.010	175	-2.264	0.025
b2.BMR.Temp. Range (56d)	-0.002	0.001	175	-2.146	0.033
b2.Tlc.Temp. Range (56d)	0.628	0.185	175	3.397	0.001
b2.Tlc.Summer	-0.574	2.979	175	-0.193	0.848
b2.Tlc.Autumn	0.758	1.913	175	0.396	0.692
b2.BMR.MitolineageB.Summer	-0.016	0.012	175	-1.360	0.176
b2.BMR.MitolineageB.Autumn	0.032	0.008	175	3.937	0.000

Evaporative water loss ($\dot{V}H_2O$) models

H1 models explained variation in evaporative water loss better than H0 models (Table A5 and A6). The best H1 models consistently included the effects of site, season, and interactions between mass × season and site × season, in addition to the expected experimental temperature and mass effects. Sex was also frequently included but was not significant in any of the best models.

Our H0 models revealed that ambient temperature, mass, short-term and medium-term weather variables, but not long-term climate variables, were good predictors of evaporative water loss in EYR. The best H0 model had an AIC value 12 higher than the best H1 model, but nonetheless produced significant estimates for all included terms: experimental temperature, mass and the weather variable summarising the minimum recorded vapour pressure in the 56 days prior to an EYR's capture. This model estimated that for each hPa decrease in minimum vapour pressure, evaporative water loss increases by about 0.21 (± 0.023). This vapour pressure variable was by far (ΔAIC 7) the best predictor in the H0 set (Table A7).

The single best H1 model of evaporative water loss in EYR showed significant effects of experimental temperature, mass, season and site (AIC: 838.95, Table A8). The largest effect after ambient temperature was mass, estimated to increase evaporative water loss by 0.096 ml H_2O min^{-1} (0.024 SE) for each gram of body weight. The effect of season was complex and included interactions with mass and site. Interaction of mass and season had the largest effect, with the general trend of increasing evaporative water loss with mass being not present in winter (i.e. lighter and heavier birds in winter had similar evaporative water loss). The interaction of site and season showed that sites differed most from each other in autumn.

To test whether season and site were good predictors of evaporative water loss (because they reflect life history rather than plastic adjustments to seasonal and spatial variation in weather and climate), we added the best predictor of the H0 models (minimal vapour pressure) to the best H1 model. The new model had a much lower AIC (814.54) than the best H0 and H1 models. Site and season remained significant, indicating that seasonal difference in evaporative water loss among sites reflects effect of variables other than minimal vapour pressure, but the difference between the Pure-Hot site and the Shared sites was no longer significant (Table 2). All other parameters remained significant and had similar estimates of the effect.

Table 2: Parameter estimates for the model combining the best H1 and H0 model of evaporative water loss (VH_2O). A random effect of individual identity of EYR was included. The AIC for this model was 814.54.

	Value	Std. Error	df	t-value	p-value
(Intercept)	1.769	0.551	301	3.211	0.002
Mass	0.062	0.024	301	2.572	0.011
SeasonSummer	-0.148	0.918	301	-0.161	0.872
SeasonWinter	1.724	0.526	301	3.279	0.001
SEXM	0.166	0.085	104	1.948	0.054
SiteShared-Cold	0.123	0.137	104	0.901	0.370
SitePure-Hot	0.116	0.190	104	0.612	0.542
SitePure-Cold	0.610	0.146	104	4.170	0.000
Min. vapour pressure (56d)	-0.192	0.037	301	-5.132	0.000
Experimental temperature	18.223	0.635	301	28.679	0.000
Experimental temperature, second order polynomial	6.672	0.603	301	11.0731	0.000
Mass:SeasonSummer	0.018	0.044	301	0.419	0.675
Mass:SeasonWinter	-0.083	0.028	301	-3.023	0.003

	Value	Std. Error	df	t-value	p-value
SeasonSummer:SiteShared-Cold	-0.067	0.230	301	-0.290	0.772
SeasonWinter:SiteShared-Cold	0.275	0.199	301	1.386	0.168
SeasonSummer:SitePure-Hot	-0.291	0.278	301	-1.047	0.296
SeasonWinter:SitePure-Hot	0.047	0.237	301	0.197	0.844
SeasonSummer:SitePure-Cold	-0.437	0.224	301	-1.946	0.053
SeasonWinter:SitePure-Cold	-0.378	0.212	301	-1.785	0.075

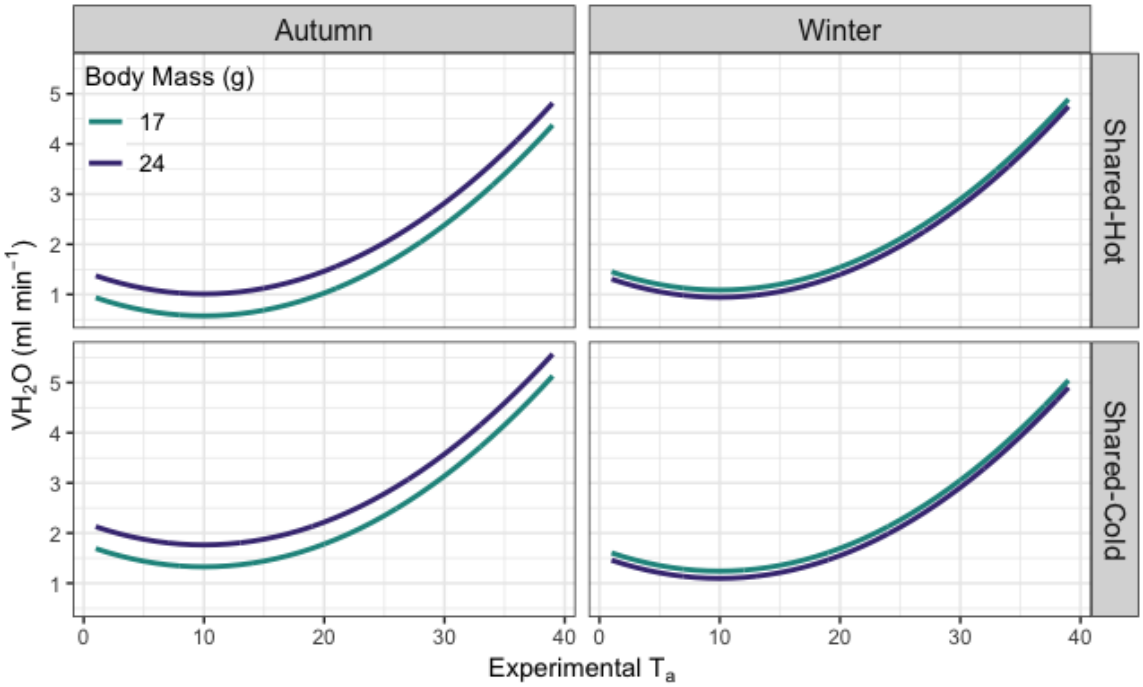

Figure 5: Effect of body mass, season and site on evaporative water loss for a light bird of 17 g (5th percentile, green) and a heavy bird of 24g (95th percentile, blue). Simulated data are based on the model summarised in Table 2, with mean values for vapour pressure, and only one sex (males, no significant differences between sexes in this model). In Autumn, heavy and light birds have significantly different rates of evaporative water loss, but they do not differ in winter. Summer did not have a significant effect on evaporative water loss, and was thus not shown.

Spatio-temporal variation in mass and weather

Two-way ANOVAs showed that birds' body mass and weather experienced prior to metabolic assessment varied between study sites and seasons. Body mass was significantly lower in both shared sites compared to the Pure-Cold site across the three seasons, while birds of the Pure-Hot site had a body mass intermediate to both (Figure 6A). Females were on average 2.8 g (±

0.4 SE) lighter than males across all sites (Table A9, Figure 6B). The interaction of season and site were not significant predictors of body mass (models not shown).

Minimum vapour pressure experienced by birds 56 days prior to capture, identified as a relevant predictor of evaporative water loss, shows the largest differences in trends across sites in autumn (Figure A2, Table A10). Autumn minimum vapour pressure was highest in the Pure-Hot, and lowest in the Shared-Hot site, which Shared-Cold and Pure-Cold being intermediate.

The temperature range experienced by EYR in the eight weeks prior to their metabolic assessment was a good predictor of metabolic rate, and varied significantly among sites and seasons (Table A11). Generally, the Pure-Cold site had the most stable temperatures (lowest temperature ranges) across all seasons, while all other sites experienced more extreme variation in temperature on average (Figure A3).

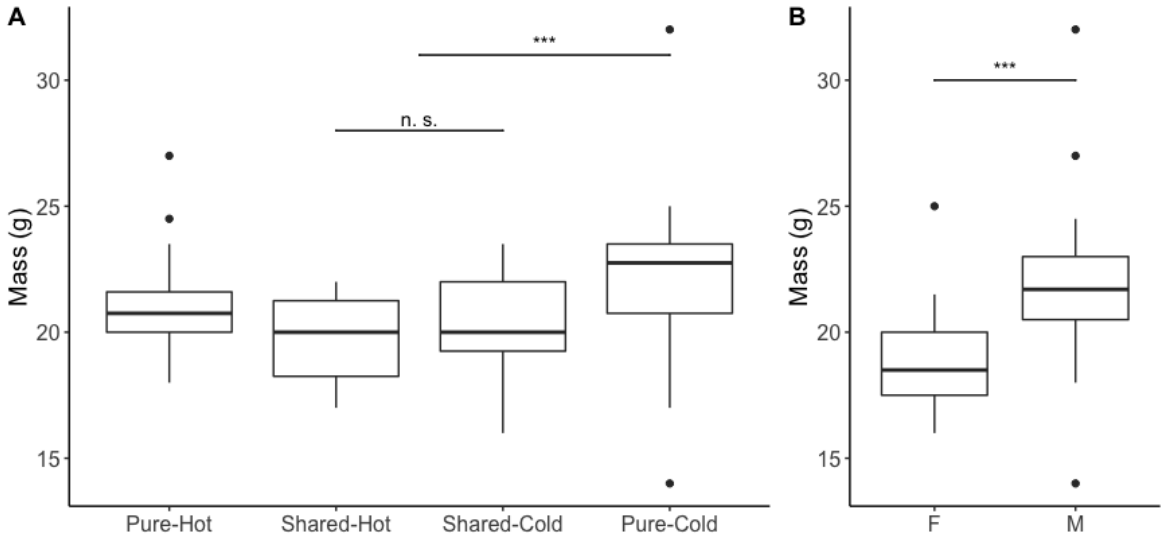

Figure 6: Body mass of EYR across sites and between sexes. A) EYR of the Pure-Cold site had the highest body mass across the assessed seasons. B) Male and Female body mass differed significantly for the pooled data of all sites and seasons.

Discussion

We investigated several aspects of energy and water metabolism in EYR to understand whether the two mitolineages convey different, and potentially climate-adaptive, metabolic phenotypes. In accordance with our predictions here and in Sunnucks *et al.* (2017), mito-A birds had a lower BMR than mito-B birds, however this was only the case in autumn. None of the other elements of energy and water metabolism investigated were predicted by mitolineage. Similar to the expectation in Sunnucks *et al.* (2017), climate variability predicted variation in metabolic rate, with the temperature range experienced in the 56 days prior to measurement showing a strong negative effect on BMR. On the other hand, evaporative water loss was best explained by minimum vapour pressure (humidity) also experienced during the 56 days prior to measurements and also showing a negative relationship.

Seasonal effects on metabolic rate

No Mitolineage Effect on BMR in Shared Sites

In the present study, metabolic rate and evaporative water loss were explained by season, even when variation in weather was accounted for. Only in autumn did mito-A individuals exhibit a lower BMR. This follows our expectations and those from Sunnucks *et al.* (2017), as we predicted that the mitolineage correlated to hotter, more arid and more variable climate to exhibit a lower BMR that is indicative of adaptation to such climate (Humphries *et al.* 2005; Naya *et al.* 2018). However, lack of significant difference between birds with different mitolineages in shared sites at any aspect of the metabolic profile fails to support the hypothesis that mitolineages convey adaptive benefits in metabolic rate. Yet, the absence of a mitolineage effect in shared sites could reflect several other factors. The environments where both mitolineages are present are not intermediate between the characteristics of the pure sites. Further, the two weather variables identified as relevant to EYR metabolism differ in autumn between shared and pure sites, with minimum vapour pressure being lower, and temperature range being higher in both shared sites compared to both pure sites. This might have caused phenotypic plasticity in metabolic traits to be stronger and to exceed genetic effects. Furthermore, at shared sites, EYR of the two mitolineages interbreed. While mtDNA is inherited unrecombined only from mothers, nuclear genome admixture would occur through inter-lineage reproduction. Mitochondrially encoded proteins work closely with nuclear encoded ones in oxidative phosphorylation. In many taxa, there is evidence that they have co-evolved to optimize its functioning (Ballard and Melvin 2010; Blier *et al.* 2012; Vijayraghavan

et al. 2019). Thus, EYR inter-lineage hybrids might have lost co-evolved mitonuclear combinations, resulting in phenotypes atypical of either pure mitolineage. In addition, these EYR inter-lineage hybrids might generally be less fit than their parental lineages, as indicated by the lower body mass of individuals from the shared sites in this current study. An increased BMR could result in lower body mass when resources are limited and was found in mito-nuclear hybrids of collared and pied flycatchers (McFarlane *et al.* 2016). To uncover whether hybridisation of the two ineages caused the loss of the metabolic phenotypes of genetically pure individuals, detailed whole-genome studies are necessary. These need to identify the mitochondrial-nuclear genotype of each EYR, link those to its mitochondrial phenotype, and compare its phenotype to a group of EYR that are considered genetically (mitochondrial and nuclear) pure. However, these studies are technically very challenging and have not yet been successfully conducted. A more common approach is to experimentally disrupt co-adapted mito-nuclear combinations by crossing genetically-diverged individuals or the replacement of the mitogenome via many generations of backcrossing. Yet, this also comes with great challenges for non-model systems (Hill *et al.* 2019).

Seasonal Effects on Evaporative Water Loss

Seasonal effects on evaporative water loss were dependent on body mass. Mass-dependence of evaporative water loss is observed in birds (Smith *et al.* 2017). However, in EYR evaporative water loss increased with mass only in summer and autumn. In winter, mass-dependence was lost, resulting in light and heavy EYR having similar rates of evaporative water loss. Evaporative water loss could be reduced in winter because of lower ambient temperatures, as variation related to humidity is already accounted for in the model. In winter EYR do not have to lose metabolic heat (which scales with body mass) because it is cold. Additionally, transpiration from respiratory organs will be lower in the cold, overall reducing water loss independently of mass, assuming the surface area of respiratory organs and bird mass do not scale strongly with each other.

The individuals of the Pure-Cold site had a generally higher evaporative water loss compared to all other sites, consistent with prior predictions for metabolic adaptation to climate (Sunnucks et al 2017). However, the strength of this effect was dependent on season and body mass. Vapour pressure was least variable at this site, which might be why an optimisation to extremes is not needed in such a stable, mesic environment (Tieleman and Williams 2002; Tieleman *et*

al. 2002; Williams and Tieleman 2005; Smit and McKechnie 2015; Smith *et al.* 2015; Gerson *et al.* 2019).

Acclimation to Medium-Term Weather Conditions

Weather variables that summarized the environmental conditions that EYR were exposed to in the 56 days prior to measurements were good predictors for basal metabolic rate, Tlc and evaporative water loss. Higher minimum vapour pressure in this period was associated with lower evaporative water loss, while greater temperature variability in this period predicted higher Tlc and lower BMR. H0 models with analogous variables summarized over shorter timescales (7d) did not explain the variation in metabolism well. In comparison, acclimation trials of three passerines to hot and cold temperatures for eight days showed an effect on BMR in only one species (Dubois *et al.* 2016). Thus, acclimation times seem to differ between species, but for EYR, this period seems to lie beyond seven days.

Negative relationship of metabolic rate and temperature range

Increase in ambient temperature is commonly linked to increases in BMR (Tieleman and Williams 2002; Williams and Tieleman 2005; Smith *et al.* 2015), however ambient temperature range has not yet been considered widely. EYR in our dataset experienced temperature variation of up to 35 °C in the 56 days prior to metabolic assessments, and our best model predicted associated decreases in BMR by up to 30%. Interestingly, the variation in temperature range across sites stems mostly from variation of the minimal temperature, not the maximum temperature experienced. However, minimal temperature was not a good predictor of metabolic rate, suggesting that neither maximum nor minimum temperatures, but instead their combined effects, shape metabolic rate in EYR. Association of metabolic rate and temperature range also fit the expectation that metabolic rate is optimized to accommodate both average minimum and average maximum environmental temperatures (Portner 2002). In EYR this might indicate that minimum temperature is a greater or more commonly experienced stressor than maximum temperatures, thus driving basal metabolic rate variation, while at the same time being balanced by the need to support less frequent high environmental temperatures.

In our best metabolic rate model, population-wide BMR increased when Tlc decreased. Tlc was also associated with temperature range experienced by the birds, increasing by 0.63 °C for every degree increase in range. This shift in Tlc in addition to the decrease in BMR supports

the emerging understanding that BMR and Tlc are dependent on each other (Fristoe *et al.* 2015; Khaliq and Hof 2018). This is based on the assumption that organisms with high BMR have higher metabolic heat production allowing them to tolerate lower temperatures (Speakman and Krol 2010).

Negative Relationship of Evaporative Water Loss and Humidity

The negative association of humidity and evaporative water observed in EYR is well documented in many species (Welch 1980; Cooper and Withers 2008; Smit *et al.* 2018). This phenomenon is commonly explained by Fick's law of diffusion (Monteith and Campbell 1980), whereby the smaller the difference in vapour concentration between the organism's body surface and the surrounding air, the lower the evaporative water loss. However, this effect is not consistent among birds (Powers 1992; Gerson *et al.* 2014; Eto *et al.* 2017; van Dyk *et al.* 2019) and no clear explanation as to why species' rates of evaporative water loss differ with humidity has emerged. EYR seem to be greatly influenced by environmental humidity, based on the observation of their evaporative water loss declining ~10-20% (depending on other factors) per hPa increase. Most studies in brids focus on long-term climatic or acute responses of evaporative water loss to humidity, but not medium- or short-term environmental humidity, so possibilities for comparisons with other studies are limited. To understand these intermediate-term relationships, ex-situ studies that acclimate individuals to different levels of humidity are needed to identify whether this response is plastic or genetically hardwired. In other species this has been done successfully but has not produced a clear picture as of yet. For example, the rodent Talas tuco-tuco did not show acclimation of EWL to different levels of humidity after a three-week acclimation period (Baldo *et al.* 2016). In contrast, offspring of Merriam's kangaroo rat parents from xeric and mesic locations raised in a common garden experiment adjusted to a similar level of EWL after a 45-day acclimation period (Tracy and Walsberg 2001).

Lack of Effect of Climate and Sex on Metabolic Rate or Evaporative Water Loss

Because mass-corrected values were used for modelling, and females are on average almost 3 g (~ 15%) lighter than males, the lack of association of metabolic rate parameters with sex was likely due to the correction used. A similar scenario is likely for evaporative water loss models, because they also included mass as a predictor in all models.

We expected that climate would explain variation in metabolic rate and evaporative water loss, because we expected the mitolineage distribution pattern of the entire range to hold true on a

smaller scale. However, climate variables had less power than weather ones to explain variation in water and energy metabolism. Climate, in particular gradients of aridity, has been identified as a major driver of metabolic rate (McNab and Morrison 1963; Tieleman and Williams 2000a; White and Seymour 2003; Withers *et al.* 2006). The effect of environmental temperature on metabolism has been shown to be latitude-dependent (Cadena *et al.* 2011; DeLong *et al.* 2018), and most studies compare species inhabiting a wide range of latitudes, which was not the case in this study of EYR. For the present study this might mean that climate variables did not show an effect on metabolic rate because our study locations did not span a latitudinal range of relevant size.

Conclusion

We estimated metabolic rate and evaporative water loss of EYR from four different natural habitats across seasons to investigate whether metabolic rate and evaporative water loss differed between the two climate-correlated EYR mitolineages. The mitolineages showed different metabolic phenotypes: mito-A EYR exhibited a lower BMR than mito-B EYR in autumn. This variation could be beneficial in climates with higher variability, such as the environments mito-A commonly inhabit, because they allow the optimizing of BMR to acute environmental conditions. However, at sites where both mitolineages were present, these seasonal phenotypes did not persist, raising the question as to whether the differences observed are based on adaptive genetic differentiation, inferior phenotypes of birds with mitochondrial-nuclear incompatibilities in the hybrid zone, or simply acclimation. We further showed that medium-term (8-week-long) temporal variation in weather and season shape metabolic rate and evaporative water loss. Variation in metabolic rate and evaporative water loss were not well explained by variation in climate, such as annual maximum temperatures, which are usually good predictors of these aspects of physiology. In summary, we provide some evidence that mitochondrial genotypes can give rise to whole-body metabolic phenotypes. However, because of the complexity of this natural system, further experimental studies are required to resolve the exact role of mitochondrial genetic variation to whole-organism metabolic phenotypes in the Eastern Yellow Robin.

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Appendix

Figures

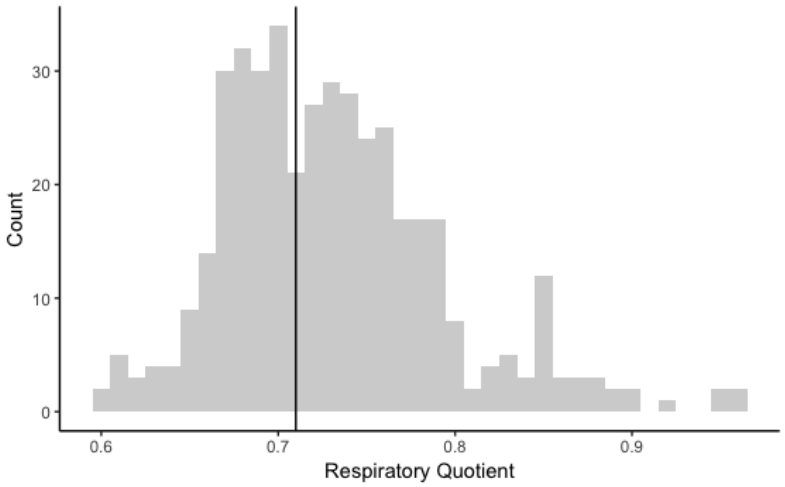

Figure A1: Frequency distribution of respiratory quotient (RQ) for all measurements (n=424) of VCO_2 and VO_2 of Eastern Yellow Robins across the four study sites and seasons (Summer - Winter). Bin size for averaging RQ estimates was 0.01. The black vertical line represents the RQ of 0.71 that is expected when Eastern Yellow Robins are post-absorptive and metabolising lipids only (Walsberg and Wolf 1995). Median RQ is 0.724, but the distribution peaks at 0.7.

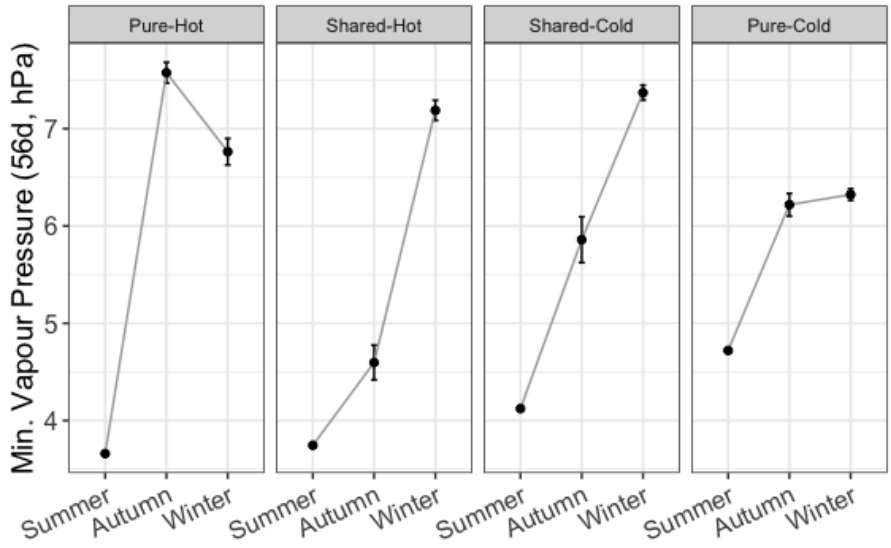

Figure A2: Seasonal and spatial variation in minimum vapour pressure experienced by EYRs in the 56 days before capture. Values are averaged per study site and season with bars representing the standard error.

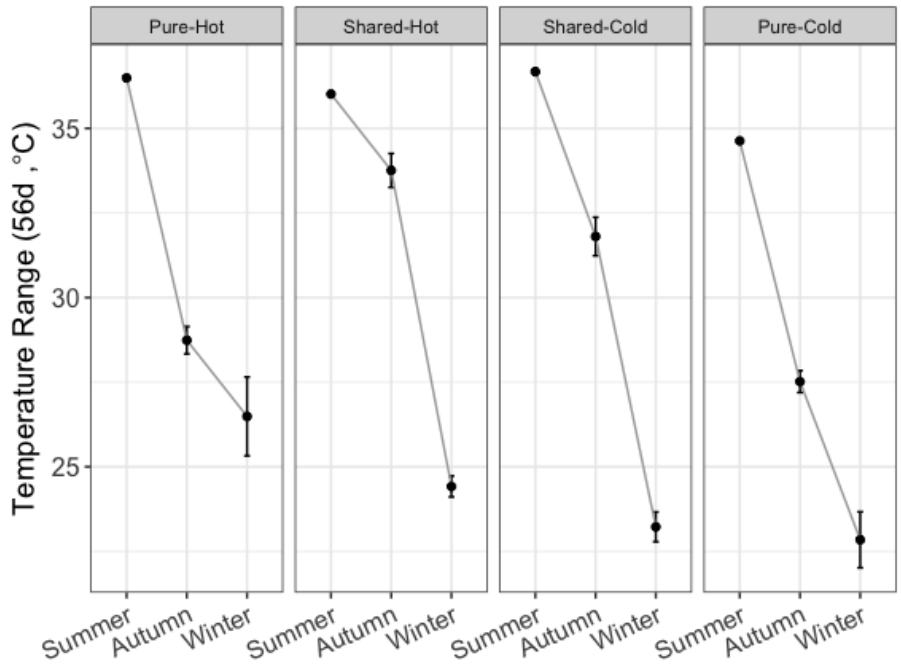

Figure A3: Seasonal and spatial variation in temperature range experienced by EYRs in the 56 days before capture. Values are averaged per study site and season with bars representing the standard error.

Tables

Table A1: The best ten H0 models explaining mass-corrected metabolic rate (VCO_2). All models are non-linear and contain two linear segments[2].

BMR	Tlc	Cmin	AIC
Temp. Range (56d)	Temp. Range (56d)		-1080.66
Temp. Range (56d)	Temp. Range (56d)	Temp. Range (56d)	-1078.76
Max. Temp (56d)	Max. Temp (56d)		-1077.68
Min. vapour pressure (56d)	Min. vapour pressure (56d)		-1077.28
Max. Temp (56d)	Max. Temp (56d)	Max. Temp (56d)	-1076.91
Min. vapour pressure (56d)		Min. vapour pressure (56d)	-1076.35
Min. vapour pressure (56d)	Min. vapour pressure (56d)	Min. vapour pressure (56d)	-1076.24
MaxT_Max07D	MaxT_Max07D		-1075.02
MaxT_Max07D	MaxT_Max07D	MaxT_Max07D	-1073.67
Temp. Range (7d)	Temp. Range (7d)		-1073.10

[2] Experimental temperature was a designated effect on all parameters, all other additional effects separated for each parameter (BMR, Tlc, and Cmin) are listed. Model quality was assessed with AIC. All models contained the random effect of individual identity of EYR on BMR.

Table A2: The best ten H1 models explaining mass-corrected metabolic rate (VCO_2)[3].

BMR	Tlc	Cmin	AIC
Season +Mitolineage×Season	Season		-1081.11
Season +Mitolineage×Season	Season	Mitolineage	-1080.56
Mitolineage + Season + Mitolineage×Season	Season		-1079.58
Season + Mitolineage×Season	Mitolineage + Season	Mitolineage	-1079.17
Mitolineage + Season + Mitolineage×Season	Mitolineage + Season	Mitolineage	-1075.43
Mitolineage + Season + Mitolineage×Season	Mitolineage + Season	Mitolineage + Season	-1073.59
Mitolineage + Season + Mitolineage×Season	Mitolineage + Season	Mitolineage + Season + Mitolineage×Season	-1072.70
Mitolineage + Season + Mitolineage×Season	Mitolineage + Season + Mitolineage×Season	Mitolineage + Season + Mitolineage×Season	-1066.17
	Site		-1065.12
Site	Site	Site	-1059.60

[3] All models contain two linear segments, one below the thermoneutral zone (TNZ, relating to the Cmin segment), and the other within the TNZ (relating to the BMR segment). Experimental temperature was a designated effect on all parameters, additional effects separated for each parameter (BMR, Tlc, and Cmin) are listed. Model quality was assessed with AIC. All models contained the random effect of individual identity of EYR on BMR.

Table A3: Parameter estimates for the best H0 model of metabolic rate (VCO_2). b1 indicates the intercept and b2 the slope of each linear compound model[4].

	Value	Std. Error	df	t-value	p-value
b1.BMR	0.244	0.017	181	14.700	0.000
b1.Tlc	1.667	3.438	181	0.485	0.628
b1.Cmin	0.006	0.001	181	9.537	0.000
b2.BMR.Temp. range (56d)	-0.002	0.001	181	-2.779	0.006
b1.Tlc.Temp. range (56d)	0.628	0.126	181	4.997	0.000

Table A4: Parameter estimates for the best H1 model of metabolic rate (VCO_2). b1 indicates the intercept and b2 the slope of each linear compound model[5].

	Value	Std. Error	df	t-value	p-value
b1.BMR	0.206	0.005	177	39.974	0.000
b1.Cmin	0.006	0.001	177	8.898	0.000
b1.Tlc	16.356	1.245	177	13.141	0.000
b2.BMR.Summer	-0.004	0.012	177	-0.356	0.722
b2.BMR.Autumn	-0.033	0.008	177	-3.843	0.000
b2.Tlc.Summer	7.034	2.066	177	3.405	0.001
b2.Tlc.Autumn	4.691	1.507	177	3.112	0.002
b2.BMR.MitolineageB.Summer	-0.017	0.012	177	-1.411	0.160
b2.BMR.MitolineageB.Autumn	0.032	0.008	177	3.956	0.000

[4] A random effect of individual identity of EYR was included on BMR. The AIC for this model was -1080.66.
[5] A random effect of individual identity of EYR was included on BMR. The AIC for this model was -1081.11.

Table A5: The best ten H1 models explaining evaporative water loss (VH_2O)[6].

Terms	AIC
Temperature^2 + Mass + Season + Site + Mass:Season + Season:Site + Sex	838.954
Temperature^2 + Mass + Season + Site + Mass:Season + Season:Site	840.449
Temperature^2 + Mass + Season + Site + Mass:Season + Season:Site + Sex + Mass:Site	840.126
Temperature^2 + Mass + Season + Site + Mass:Season + Season:Site + Sex + Mitolineage	840.610
Temperature^2 + Mass + Season + Site + Mass:Season + Season:Site + Sex + Mass:Sex	840.742
Temperature^2 + Mass + Season + Site + Mass:Season + Season:Site + Mitolineage	842.120
Temperature^2 + Mass + Season + Site + Mass:Season + Season:Site + Mass:Site	841.804
Temperature^2 + Mass + Season + Site + Mass:Season + Season:Site + Sex + Mitolineage + Mitolineage:Sex	841.940
Temperature^2 + Mass + Season + Site + Mass:Season + Sex + Mitolineage + Mitolineage:Season	843.056
Temperature^2 + Mass + Season + Site + Mass:Season + Season:Site + Sex + Mitolineage + Mass:Site	841.699

[6] All models fitted a second order polynomial on ambient temperature. All main effects and interactions are listed. Model quality was assessed with AIC. All models contained the random effect (intercept only) of individual identity of EYR.

Table A6: The best ten H0 models explaining evaporative water loss (VH_2O)[7].

Basic term	Varying term	AIC
Temperature^2 + Mass +	Min. vapour pressure (56d)	851.191
Temperature^2 + Mass +	Days without rain (7d)	858.984
Temperature^2 + Mass +	Lowest min. temp (56d)	865.698
Temperature^2 + Mass +	Temp. Range (7d)	868.380
Temperature^2 + Mass +	Temp. Range (56d)	872.679
Temperature^2 + Mass +	MinT_Min07D	885.937
Temperature^2 + Mass +	MinT_Min07D	885.937
Temperature^2 + Mass +	VapP09_Min07D	900.113
Temperature^2 + Mass +	VapP15_Mean56D	905.619
Temperature^2 + Mass +	Historical evaporation in August (30yr)	918.092

Table A7: Parameter estimates for the best H0 model of evaporative water loss (VH_2O). A random effect of individual identity of EYR was included. The AIC for this model was 851.191.

	Value	Std.Error	df	t-value	p-value
(Intercept)	2.773	0.340	311	8.157	0.000
Min. vapour pressure (56d)	-0.210	0.023	311	-8.969	0.000
Mass	0.036	0.014	311	2.512	0.013
Temperature^2 (1)	18.353	0.648	311	28.309	0.000
Temperature^2 (2)	6.561	0.612	311	10.724	0.000

[7] All models fitted a polynomial of the second order on ambient temperature and included mass as a main effect. All main effects and interactions are listed. Model quality was assessed with AIC. All models contained the random effect (intercept only) of individual identity of EYR.

Table A8: Parameter estimates for the best H1 model of evaporative water loss (VH_2O)[8].

	Value	Std. Error	df	t-value	p-value
(Intercept)	0.110	0.467	302	0.236	0.814
SeasonSummer	0.658	0.954	302	0.690	0.491
SeasonWinter	1.921	0.549	302	3.500	0.001
SexM	0.169	0.091	104	1.861	0.066
SiteShared-Cold	0.019	0.143	104	0.135	0.893
SiteShared-Hot	-0.419	0.168	104	-2.498	0.014
SitePure-Cold	0.391	0.147	104	2.653	0.009
Mass	0.097	0.024	302	3.952	0.000
Temperature^2 (1)	18.124	0.647	302	28.000	0.000
Temperature^2 (2)	7.042	0.614	302	11.468	0.000
SeasonSummer:Mass	-0.007	0.046	302	-0.149	0.882
SeasonWinter:Mass	-0.110	0.028	302	-3.923	0.000
SeasonSummer:SiteShared-Cold	-0.106	0.242	302	-0.440	0.660
SeasonWinter:SiteShared-Cold	0.268	0.210	302	1.280	0.202
SeasonSummer:SiteShared-Hot	0.206	0.273	302	0.754	0.452
SeasonWinter:SiteShared-Hot	0.572	0.224	302	2.557	0.011
SeasonSummer:SitePure-Cold	-0.491	0.234	302	-2.099	0.037
SeasonWinter:SitePure-Cold	-0.097	0.215	302	-0.451	0.653

Table A9: Results of a two-way ANOVA of EYR body mass as a function of season and site (no interaction).

	df	Sum Sq	Mean Sq	F value	P value
Season	3	108.100	36.030	10.120	0.000
Site	1	201.400	201.390	56.590	0.000

[8] A random effect of individual identity of EYR was included. The AIC for this model was 838.95.

Table A10: Results of a two-way ANOVA of minimal vapour pressure experienced by EYR in the 56 days prior to metabolic assessment.

	df	Sum Sq	Mean Sq	F value	P value
Season	2	114.140	57.070	63.696	0.000
Site	3	13.410	4.470	4.988	0.003
Season:Site	6	49.520	8.250	9.211	0.000

Table A11: Results of a two-way ANOVA of temperature range experienced by EYR in the 56 days prior to metabolic assessment.

	df	Sum Sq	Mean Sq	F value	P value
Season	2	2193.100	1096.600	79.118	0.000
Site	3	296.200	98.700	7.125	0.000
Season:Site	6	243.600	40.600	2.930	0.011

CHAPTER 4

Blood Cell Respiration predicts Basal Metabolic Rate

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Abstract

Endotherms living in cold climates and at high latitudes typically have higher metabolic rates than those living in warmer climates and low latitudes. The metabolic rate of an individual is modulated by the cellular respiration of its organs and tissues, which in turn are modulated by mitochondrial function. Mitochondrial genetic variation might therefore represent the proximate cause of climate adaptive metabolic phenotypes, acting via mitochondrially encoded proteins crucial for mitochondrial function.

We investigated the links between mitochondrial genetic variation and metabolic phenotypes of the mitochondria, cell, and the whole organism in an Australian passerine, the Eastern Yellow Robin (EYR). EYR harbours two mitochondrial lineages (mitolineages) that exhibit strong associations with climate and encode different protein variants involved in mitochondrial function; the 'inland' lineage (mito-A) is found in hotter and more variable environments compared to the more stable, cooler conditions of the 'coastal' lineage (mito-B). In previous studies we showed while 1) mitochondrial function was more strongly predicted by climate and weather than by mitolineage, but that nonetheless 2) individuals of the inland mitolineage had lower resting metabolic rates in autumn than birds of the coastal mitolineage.

Here, for 37 birds for which blood and whole-organism metabolism were assessed on the same occasion, we tested whether cellular and mitochondrial metabolism predict that of the resting individual. We show that among-individual variation in cellular metabolism of intact blood cells powered by endogenous nutrient supply (*ROUTINE* respiration) explained one third of the among-individual variation in basal metabolic rate. Then, using structural equation models, we show that birds with higher body mass and *ROUTINE* respiration, and which had experienced narrower range of temperatures, had higher basal metabolic rates. Mitolineage, on the other hand, did not directly predict resting metabolic rates of these 37 birds.

The climate-correlated distribution of the two mitolineages, together with the support for mitochondrial function modulating whole-organism resting metabolic rates, point to mitochondria as mediators of climate adaptive metabolic responses. However, to what extent mitochondrial genetic variation in EYR contributes to climate adaptation of mitochondrial functions requires manipulative studies.

Keywords*:* [1] Bioenergetics, [2] ROUTINE respiration, [3] Eco-physiology, [4] Aves, [5] Mitochondrial function, [6] Climate adaptation

Introduction

Endothermic animals are adapted to their climate by many different mechanisms, including those responsible for maintaining body temperature and efficiently utilising energy available from nutrients. The most studied metabolic parameter in this context is the energy an organism uses when not expending excess energy on digestion, reproduction, physical activity and thermoregulation, called basal metabolic rate (BMR) in endotherms. BMR is typically lower in species found in more tropical and warmer regions (Speakman and Rowland 1999; Williams and Tieleman 2000; Lovegrove 2003; Rezende *et al.* 2004; White *et al.* 2007a; Naya and Bozinovic 2012; Stager *et al.* 2016).

Climate, together with body mass, is a consistent predictor of variation in BMR among species (White and Kearney 2013b, 2014). Within species, however, there is significant variation in BMR despite limited variation in mass (Norin and Metcalfe 2019). This variation is repeatable (White *et al.* 2013; Auer *et al.* 2016) and heritable (Pettersen *et al.* 2018a), suggesting that processes intrinsic to individuals determine BMR. What exactly these physiological processes are has been debated for decades (Brand 1990; Wheatley and Clegg 1994; Konarzewski and Książek 2013; White and Kearney 2013a; Norin and Metcalfe 2019). One prominent explanation for intraspecific variation in metabolic rates is that variation in whole-animal metabolism is driven by variation in mitochondrial metabolism (Porter *et al.* 1996; Rolfe and Brand 1997a; Dulloo and Samec 2001; White and Kearney 2013a, 2014). The oxidative phosphorylation pathway in the mitochondria is the main provider of cellular energy. It supplies ~90% of the energy needed to fuel cellular processes, by using nutritional energy to phosphorylate ADP to ATP (Rolfe and Brand 1997b). Consequently, changes in oxidative phosphorylation have the potential to convey metabolic adaptation to climate. So far, studies trying to link variation in oxidative phosphorylation to whole-organism metabolic rate have been mostly inconclusive (Norin and Metcalfe 2019).

Here, in an Australian passerine, the Eastern Yellow Robin (EYR, *Eopsaltria australis*), we study the role of mitochondria in conveying climate-adaptive whole-organism phenotypes. This species has two climate-correlated mitochondrial lineages (mitolineages, 'inland' mito-A and 'coastal' mito-B), that are allopatric for most of the species' range, despite nuclear gene flow (Pavlova *et al.* 2013; Morales *et al.* 2017), with narrow hybrid zones where individuals of the two mitolineages come into contact. Mitochondrial genetic variation between the mitolineages shows signatures of positive selection and is hypothesized to convey climate adaptation via

differences in tertiary structure of oxidative phosphorylation proteins (Morales *et al.* 2015; Sunnucks *et al.* 2017; Lamb *et al.* 2018). In two previous studies we showed that 1) that mitochondrial function was more strongly predicted by climate and weather than mitolineage (Chapter 2), nonetheless 2) in autumn, individuals of the inland mitolineage had a lower BMR than coastal mitolineage birds (Chapter 3). However, to fully understand the role of mitochondria in conveying climate-adaptive whole-organism phenotypes, we need to establish whether mitochondrial function modulates BMR.

Here, we analysed data on mitochondrial function and whole-organism BMR collected from the same EYR individuals on the same day to test to what extent mitochondrial function explains variation in BMR. We also include previously-identified candidate environmental drivers of mitochondrial and whole-organism metabolism in EYR (Chapters 2 and 3) to construct a framework of mitochondria-mediated climate adaptation in EYR, as follows. Within the individual, mitochondrial genetic variation (mtDNA) conveys differences in mitochondrial function. These are likely conveyed via altered proteins that change the physio-chemical properties of oxidative phosphorylation, improving the efficiency of ATP production and thus reducing the amount of energy released as heat per unit ATP produced in individuals of the mito-A (inland) lineage. Such a system is referred to as 'highly coupled', because per unit of energy input there is a large amount of ATP produced. On the other hand, individuals of the mito-B (coastal) lineage are expected to have lower coupling, thus releasing more heat and producing less ATP per unit of nutrients input. This would be an adaptive mechanism because mito-B birds live in cooler, less variable environments in which maintaining a constant body temperature is expected to require greater rates of heat production. Mito-B birds are also at lower risk of hyperthermia and likely live in an environment where food and water are less limiting than in the typical environments of mito-A EYR (Sunnucks *et al.* 2017). BMR is expected to be lower in mito-A individuals, because a more efficient oxidative phosphorylation system will produce less excess heat at the thermoneutral temperature range over which BMR is measured (Figure 1).

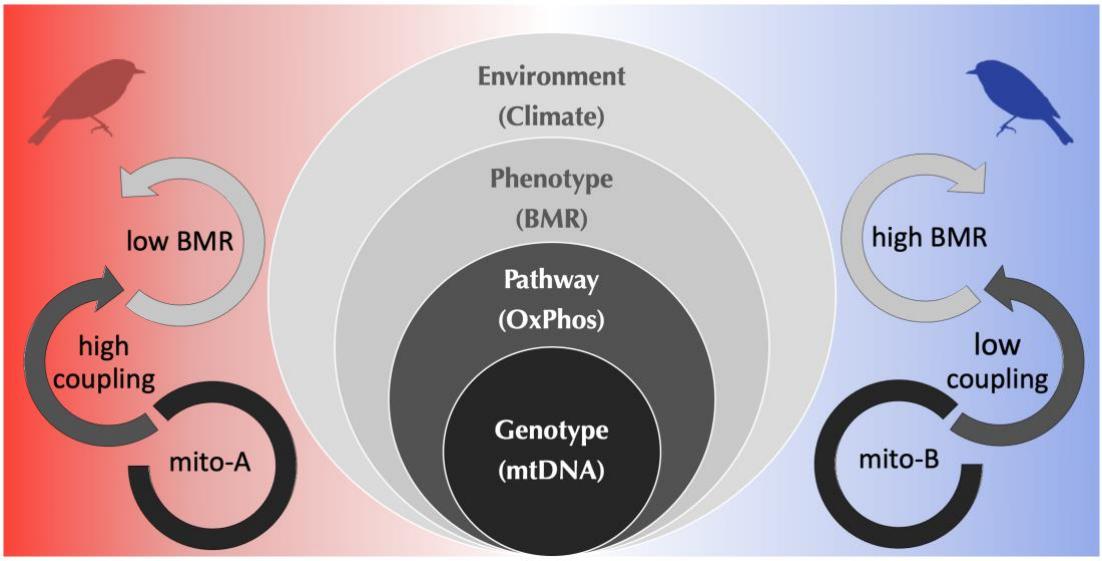

Figure 1: Framework of mitochondria-mediated climate adaptation (based on Sunnucks et al. 2017). The central section depicts the levels of biological organization involved, each contained within a higher level, until the level 'environment' is reached. Within the individual, mitochondrial genetic variation (mtDNA) causes differences in mitochondrial function, likely via altered proteins of the oxidative phosphorylation pathway (OxPhos). This variation carries through to the level of the entire organism, where mitochondrial function affects basal metabolic rate (BMR). Metabolic rate of the individual is a trait on which selection can then act. For individuals of the mito-A lineage that are commonly found in 'inland' hotter, more arid and more variable environments, it is expected that mtDNA conveys more-coupled OxPhos system, which in turn conveys lower BMR.

Methods

Field sampling, genetic and metabolic assessment

The study was conducted in South-Eastern Australia, between June 2017 and July 2018. Four study sites were chosen to present subtle differences in climate, represented by maximum summer and minimum winter temperatures. At two sites of contrasting climates, referred to as Pure-Hot and Pure-Cold, EYR of only a single mitolineage (mito-A and mito-B, respectively) were present. At the other two sites, referred to as Shared-Hot and Shared-Cold, EYR of both mitolineages were present, with documented cases of interbreeding between them (Walters, unpublished Honours thesis, Monash University). We caught wild EYR with mist-nets or spring traps, then colour-banded, measured, aged, assessed moult, and took a blood sample (<75 µl) for genetic analyses (Chapters 2 and 3). Sex and mitolineage (mito-A or mito-B) of each individual were determined using sexing PCR and sequences of the mitochondrial ND2 gene (Genbank accession pending), as detailed in Pavlova *et al.* (2013).

Whole-organism respirometry experiments took place between March 2017 and July 2018. We purposefully excluded measurements during the EYR breeding season (August - December) to avoid disrupting breeding of individuals that were subjects in other long-term fitness studies. Whole-organism metabolic rate was measured on the night following capture, using standard positive-pressure open-flow multi-channel respirometry (Chapter 3). EYR were released at the site of capture at sunrise following measurements. We chose to focus on BMR because it is a widely studied heritable trait (Pettersen *et al.* 2018b), and has been linked to climate variation (Tieleman and Williams 2000b; Stager *et al.* 2016). We selected BMR measures from the metabolic rate data measured across the range of temperatures in Chapter 3, by including only measures that fell within the estimated thermoneutral zone, i.e. between the estimated season-specific lower critical temperature (Summer: 23 °C, Autumn: 21 °C, Winter: 16 °C) and the estimated upper critical temperature 28 °C of the thermoneutral zone. Rates of CO_2 production ($\dot{V}CO_2$, ml CO_2 min^{-1}) were used to quantify metabolic rate (Chapter 3).

For 33 of 37 individuals, a blood sample taken on the morning after whole-organism respirometry was used to assess mitochondrial function of blood cells using high-resolution respirometry (O2k, Oroboros Austria) (see Chapter 2 for details). For the remaining four individuals, mitochondrial function was assessed during the day of capture, at least two hours before whole-body experiments took place. These were individuals that had mitochondrial

respiration assessed earlier in the day but were re-captured later the same day and then included in whole-body respirometry. These four occasions were randomly distributed throughout the sampling period and they did not show a statistically different blood cell or whole-body metabolism. *ROUTINE* respiration was measured in intact blood cells, while all other states were measured after permeabilization of the cell membrane and with saturating concentrations of endogenous substrate. *COMPLEX I* respiration rate was achieved by adding substrates fuelling sugar metabolism and feeding electrons into Complex I only, *OXPHOS* respiration was achieved by adding substrates of Complex II, *LEAK* respiration was achieved by adding a Complex V inhibitor. The parameters of mitochondrial function tested as predictors of BMR were: rates of oxygen consumption (oxygen flux rates [pmol O2 mg^{-1} protein sec^{-1}]) of four states (*ROUTINE, COMPLEX I, OXPHOS, LEAK*), and relevant coupling control ratios (*LEAK/OXPHOS, LEAK/ROUTINE, ROUTINE/OXPHOS*) as well as free capacities: *free OXPHOS* (*OXPHOS* without *LEAK* proportion), *free ROUTINE* (*ROUTINE* without *LEAK* proportion) (Gnaiger *et al.* 2000). As the quantity of tissue used in the assays varied, the respiration rates were corrected for protein concentration, which provided an estimate of cell quantity (Chapter 2).

Our final dataset contained assessments of 37 individuals for mitochondrial metabolism and BMR taken within hours of each other. These were distributed over four study sites, two mitolineages and three seasons (Table 1).

Table 1: Distribution of individuals assessed for mitochondrial function and BMR and used to infer their relationship to each other in the current work.

Study Site	Mitolineage	Number of individuals
Pure-Cold	mito-B	10
Shared-Cold	mito-A	4
Shared-Cold	mito-B	9
Shared-Hot	mito-A	9
Shared-Hot	mito-B	2
Pure-Hot	mito-A	3

Modelling relationships between mitochondrial function and BMR

Nine models were built of the relationship between mitochondrial function and BMR (a single measure of mitochondrial function was used as a predictor in each). Linear models did not

meet the criterion of normally distributed residuals. Thus, we used generalised linear mixed models with gamma error distribution and log-link function, plus a logit-transformed $\dot{V}CO_2$ as response. All models included body mass because we were interested in explaining mass-independent variation in BMR with parameters of mitochondrial function. All statistical analyses were carried out in R (R Core Team 2018) and (RStudio Team 2016).

Structural Equation Models

Structural equation modelling (SEM) allows us to assess a system of models in a defined framework. Response variables of one model can be integrated in other models as predictors and so connect different models. We used piecewise SEM (Lefcheck 2016) to implement these models in a framework. In our case, we combined models from previous studies by using mitochondrial function as a predictor of BMR. Apart from this, another main benefit in using SEM in our system is that we can identify indirect effects of predictors of mitochondrial function on BMR. First, we built two connected models (Figure 2): 1) a model predicting mitochondrial function and 2) a model predicting BMR. These were connected by the mitochondrial function parameter found to predict BMR. The three predictors in the mitochondrial function model were 1) average vapour pressure experienced by EYR in the 56 days prior to mitochondrial assessment, and the 2) number of days without rainfall in the 7 days prior to assessment, as well as 3) a term for normalization of assay input as identified in Chapter 2. The second model, predicting BMR, included five predictors: 1) the relevant parameter of mitochondrial function, 2) ambient temperature range (56 days prior to capture), 3) mitolineage, 4) season (Summer, Winter, Autumn) and 5) body mass, as identified in Chapter 3. We used Fisher's C and associated Chi-squared test to assess whether the model explained the relationships in the system well (a model is rejected if $p < 0.05$).

We then simplified this model framework of two connected models by stepwise removal of non-significant effects until the overall model quality was maximised, as assessed by the Akaike information criterion corrected for small sample sizes (AICc). The model of the lowest AICc score was taken to be the one that explains relationships in the system, given the data best.

We next compared the predictors of BMR in our best framework with temperature variables commonly linked to metabolic rate, in particular annual maximum, mean and minimum temperature (Withers *et al.* 2006; Jetz *et al.* 2007; White *et al.* 2007a; Withers and Cooper 2014; Naya *et al.* 2018). To achieve this, we simply replaced the temperature range term in the best framework for maximum and minimum temperature experienced also for 56 days prior to

metabolic assessment, respectively. To more easily compare the resulting models, we returned them into a gaussian framework, and also corrected mitochondrial respiration rates for protein concentration, turning them from frameworks to simple linear models. These models passed visual quality checks, confirming normality of residuals. The quality of these simplified models was compared using AIC.

Results

Mitochondrial *ROUTINE* Respiration predicts BMR

Of eight measures of mitochondrial function tested as predictors of BMR, only one (*ROUTINE*) showed a significant predictive power (Table 2). The generalised linear model (with gamma error distribution and log-link function) showed positive relationships of body mass and *ROUTINE* respiration with BMR, such that a unit increase in BMR is associated with a 1.136-fold increase in *ROUTINE* respiration.

Table 2: Summary of all model parameter estimates for each of the models predicting BMR with one of the nine mitochondrial function parameters plus body mass. The only parameter of mitochondrial function that showed a significant association with BMR was ROUTINE respiration (bold).

Model term	Estimate	Std. Error	t value	p value
Intercept	-3.847	0.972	-3.957	0.000
ROUTINE	127.396	52.634	2.420	0.021
Body Mass	0.149	0.041	3.618	0.001
Intercept	-2.838	1.126	-2.520	0.017
COMPLEX I	1.764	14.302	0.123	0.903
Body Mass	0.136	0.048	2.841	0.008
Intercept	-3.338	1.087	-3.070	0.004
OXPHOS	7.163	7.727	0.927	0.360
Body Mass	0.148	0.047	3.154	0.003
Intercept	-2.952	1.030	-2.864	0.007
LEAK	22.280	39.636	0.562	0.578
Body Mass	0.134	0.045	2.944	0.006
Intercept	-2.764	0.981	-2.817	0.008
LEAK/OXPHOS	-0.673	1.215	-0.554	0.583
Body Mass	0.142	0.047	3.009	0.005
Intercept	-2.831	0.976	-2.901	0.006
ROUTINE/OXPHOS	0.873	1.748	0.499	0.621
Body Mass	0.131	0.046	2.861	0.007

Model term	Estimate	Std. Error	t value	p value
Intercept	-2.789	0.961	-2.901	0.006
LEAK/ROUTINE	-57.025	184.087	-0.310	0.759
Body Mass	0.138	0.047	2.941	0.006
Intercept	-3.465	1.130	-3.065	0.004
Free OXPHOS	9.408	9.401	1.001	0.324
Body Mass	0.154	0.048	3.180	0.003
Intercept	-2.794	0.970	-2.882	0.007
Free ROUTINE	44.145	54.384	0.812	0.423
Body Mass	0.141	0.046	3.037	0.005

Framework testing with Structural Equation Models

The framework describing energy metabolism in EYR developed based on previous findings (Chapters 2 and 3) was supported by the metabolic and environmental data. (Fisher's C = 16.893, df=14, p=0.262). Positive significant effects were identified for body mass on BMR, and protein concentration on *ROUTINE* respiration (Table 3, Figure 2a).

The framework of highest quality did not include the effect of mitolineage and season on BMR, and vapour pressure and days without rain on *ROUTINE* respiration (AICc = 32.633, Fisher's C = 5.813, df = 4, p = 0.214, Figure S1). In the simplified version of this framework (linear model of BMR, *ROUTINE* corrected for protein concentration), ROUTINE respiration remained a significant predictor of BMR, with BMR increasing by 0.291 standard deviations per each standard deviation increase in *ROUTINE* respiration (Figure 2b, Table 4). Body mass has the strongest association with BMR in this model, with a standardized effect size of 0.54, followed by a negative effect of temperature range with a standardized effect size of -0.345.

Table 3: Model coefficients for the original structural equation model implementing effects identified in Chapter 2 and 3, and in the current work. The bottom part of the table gives estimates for the relationships we defined as independent[9].

Response	Predictor	Estimate	Std. error	df	Crit. Value	p value
ROUTINE	Vapour pressure (3pm, 56d)	0.026	0.013	33	1.940	0.061
ROUTINE	Days no rain (7d)	-0.007	0.005	33	-1.528	0.136
ROUTINE	Protein concentration	-0.004	0.001	33	-7.144	0.000
logitVCO2	Mass	0.145	0.044	27	3.330	0.003
logitVCO2	Temperature range (56d)	-0.075	0.043	27	-1.755	0.091
logitVCO2	Autumn	-0.052	0.378	27	-0.137	0.892
logitVCO2	Winter	-0.921	0.682	27	-1.350	0.188
logitVCO2	Mito-B	-0.276	0.320	27	-0.863	0.396
logitVCO2	ROUTINE	0.101	0.060	27	1.669	0.107
logitVCO2	Winter:Mito-B	0.208	0.404	27	0.516	0.610
logitVCO2	Autumn:Mito-B	-0.240	0.486	27	-0.495	0.625
logitVCO2	ROUTINE:Protein conc.	-0.003	0.002	27	-1.573	0.127
Independ Claim						
logitVCO2 ~ Vapour pressure (3pm, 56d)		0.028	0.793	26	0.035	0.972
logitVCO2 ~ Days no rain (7d)		0.222	0.092	26	2.414	0.023
ROUTINE ~ Mass		0.004	0.002	32	2.027	0.051
ROUTINE ~ Temperature range (56d)		0.001	0.002	32	0.323	0.749
ROUTINE ~ Autumn		-0.016	0.018	32	-0.872	0.390
ROUTINE ~ Winter		-0.007	0.020	32	-0.327	0.746
ROUTINE ~ Mito-B		-0.001	0.011	32	-0.130	0.897

Table 4: Parameter estimates of scale-standardised data for the best structural equation model that was simplified into a linear model (Figure 1b). The estimates for the relationships we identified as independent were all not significant.

Term	Estimate	Std. Error	t value	p value
Intercept	0.000	0.133	0.000	1.000
Mass	0.542	0.139	3.915	0.000
Temperature range (56d)	-0.345	0.136	-2.543	0.016
ROUTINE (corrected for protein concentration)	0.291	0.138	2.106	0.043

[9] Independent claim: a relationship of variables in the framework that were defined as independent but were tested for independence and used to estimate Fisher's C.

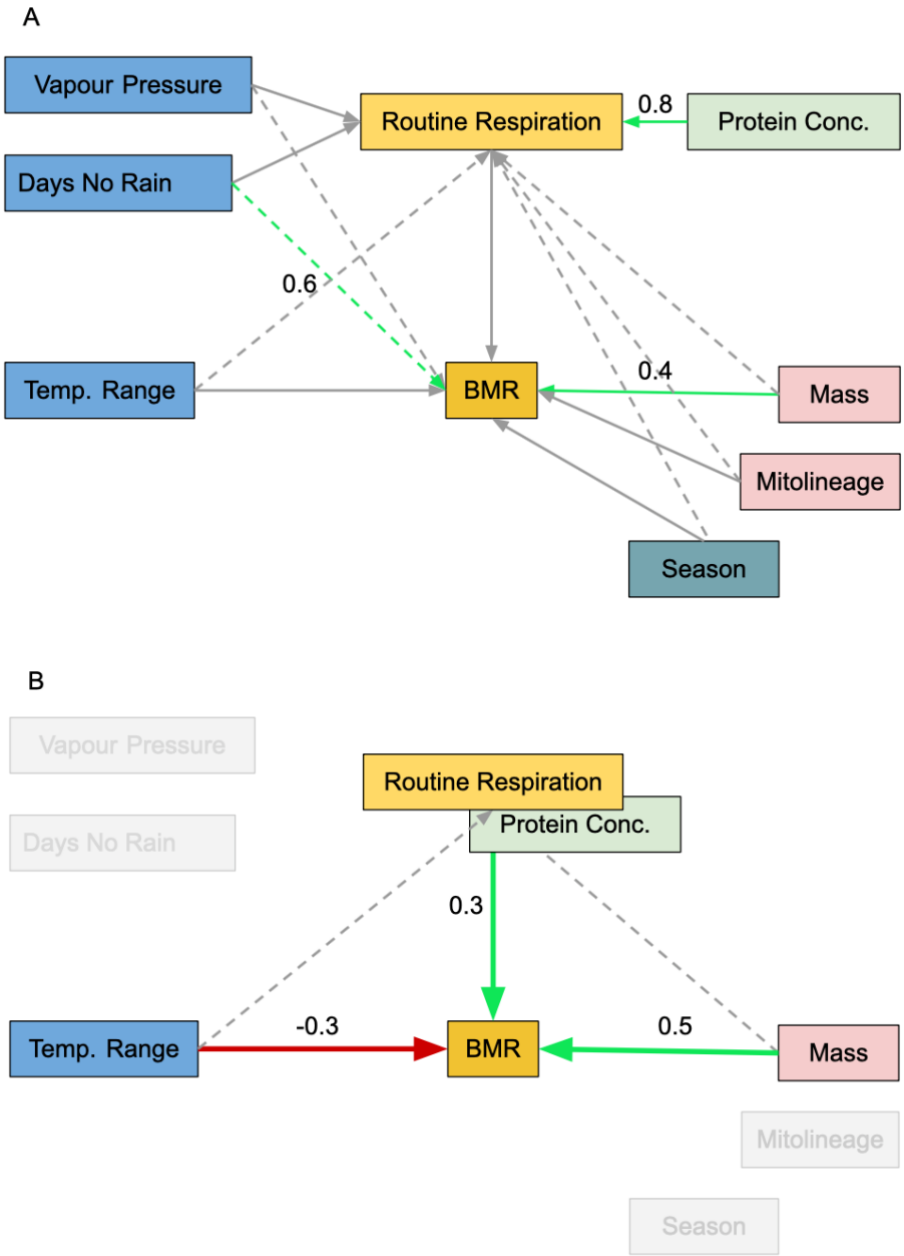

Figure 2: Modelled relationships of energy metabolism in Eastern Yellow Robin (EYR). Solid arrow lines show inferred relationships identified by previous work (Chapters 2 and 3, except for ROUTINE respiration on BMR) and in the current study, dashed arrows indicate additional relationships tested in the framework. Grey arrows denote non-significant relationships, red arrows indicate negative relationships, and green arrows indicate positive relationships. Numbers represent the scaled effect sizes. A: Original framework; weather conditions experienced by EYR in the 56 days before metabolic assessment, influence ROUTINE respiration. BMR is furthermore affected by body mass, the temperature range experienced by individuals in the 56 days before metabolic assessments, mitolineage and season. B: The best framework after simplification into a single linear model. Compared to original framework (A), it excludes the association of weather with

ROUTINE respiration, and of season and mitolineage with BMR (greyed out boxes without arrows).

Comparison of Temperature Range, Minimum Temperature, and Maximum Temperature as drivers of BMR

As in the larger dataset (Chapter 2), temperature range predicted BMR marginally better than did minimum or maximum temperatures in the best framework. Quality was almost identical for the models including either temperature range, maximum or minimum temperature (AICc = 32.633, 32.501, 32.238, respectively). In particular, temperature range and maximum temperature carried almost the same information (Figure S2), and explained similar proportions of variation in the BMR models (R_2: 0.41 and 0.40, respectively, Table S1). Minimum temperature on the other hand explained slightly less variation in BMR (R_2 = 0.37, Table S2).

Discussion

Blood cell ROUTINE respiration predicts BMR

Our data suggest that cellular mechanisms rather than mitochondrial respiration alone drives BMR in the Eastern Yellow Robin. *ROUTINE* respiration was the only measure of mitochondrial metabolism that showed significant association with BMR. During *ROUTINE* respiration the cell experiences conditions most similar to those in situ, as the cell is intact. All other measures were achieved after cells were permeabilized to enable direct interaction of mitochondria with various substrates. No other measure than *ROUTINE* had a significant relationship with BMR. This includes all mitochondrial respiration rates, which, if significant, would indicate that mitochondrial activity rates (or mitochondrial density) were impacting BMR. Other attributes of oxidative phosphorylation, in particular coupling, also showed no evidence of impacting BMR.

The use of isolated mitochondria and permeabilized cells might not be appropriate for studying the relationship between mitochondrial function and BMR. In our mitochondrial assays, after permeabilization of the cells and mitochondria, we physiologically saturated substrate concentrations, which might have led to oxygen consumption rates unrepresentative of intact living systems. Rather than representing rates of minimal metabolism, as does BMR, these substrate-saturated mitochondrial respiration rates might be more relevant for maximum metabolic rates of active individuals, which we did not assess in EYR (Jacobs *et al.* 2012; Coen *et al.* 2013; Schlagowski *et al.* 2014; Salin *et al.* 2016).

To the best of our knowledge, no other study has linked *ROUTINE* respiration to BMR. This might be due to the preference for studying isolated mitochondria rather than intact and permeabilised cells. In other species, such as rats, possums, and frogs, mitochondria- rather than cell-specific rates of respiration, in particular *LEAK* respiration (reflecting metabolic heat production, rather than ATP), show a positive relationship with BMR (Rolfe and Brand 1997b; Polymeropoulos *et al.* 2012; Salin *et al.* 2012). However, the generality of this relationship is questionable, because many other studies have not found associations between any mitochondrial function parameters and whole-organism metabolic rates (Glazier 2015; Polymeropoulos *et al.* 2017; Norin and Metcalfe 2019).

The use of blood to study mitochondrial function, instead of tissue, is particularly beneficial in study systems where repeated measures of the same individuals are required, such as studies on

aging and long-term fitness (Stier *et al.* 2013; Stier *et al.* 2015). We demonstrated that blood cell respiration does significantly predict a relatively large amount (~30%) of variation in EYR basal metabolic rate. This strong correlation, plus the use of respiration rates of peripheral blood mononuclear cells in human studies of mitochondrial dysfunction and declines in energy metabolism, show that blood is an important alternative to other tissues in energy-related studies (Gubert *et al.* 2013; Karabatsiakis *et al.* 2014).

Cellular respiration explains BMR

All frameworks summarising the effects of various predictors on BMR in Eastern Yellow Robin did not show mitolineage as a significant driver of BMR variation. Instead, they highlighted the effects of mitochondrial *ROUTINE* respiration, mass, and temperature range experienced by the birds prior to assessment as drivers of variation in BMR. However, all frameworks, even the original framework containing all predictors identified in previous studies, were supported (Fischer's $C > 0.05$), in turn supporting the findings of Chapters 2 and 3. The best framework explained 49% of the variation in BMR. High inter-individual variation in BMR is common (Norin and Metcalfe 2019), and thus the amount of unexplained variation in our BMR models is unsurprising. Our data did not include repeated measurements of the same individuals, so that we cannot assess the extent of intra-individual repeatability, which would be required to make further statements about other drivers of variation in BMR.

EYR do not show evidence that they use mass or *ROUTINE* respiration to adjust their BMR to the temperatures they experienced. If this was the case, temperature range should not be independent of either of these traits, which it was in this study. Thus, other physiological mechanisms must enable birds to adjust their BMR in response to the temperature they experience. Changes in body composition, in particular relative organ sizes, could explain mass- and *ROUTINE* respiration-independent changes in BMR to cope with variation in temperature (Vezina *et al.* 2006; Wang *et al.* 2012; Glazier 2015).

Comparison of Temperature Range, Minimum Temperature, and Maximum Temperature as drivers of BMR

Temperature range was a slightly stronger predictor of BMR compared to minimum and maximum temperature, but the quality of the different temperature models was nearly identical. However, the variation explained by the BMR model, the precision of the parameter estimates, and standardized effect sizes were higher when temperature range was included, and

not maximum or minimum temperature. This re-iterates previous findings based on more than three times these sample sizes, in which temperature range was a better predictor of BMR in EYR than were minimum and maximum temperature (Chapter 3). Furthermore, the effect of the temperature range estimated in this dataset was within the 95% confidence interval of the estimate in Chapter 3 (-0.002 ± 0.001 SE), with a decrease in BMR of 0.006 ml CO_2/min (±0.002 SE) for every degree of increases temperature range. This is consistent with the hypothesis that metabolic rate is optimized according to temperature range experienced rather than just one of its extremes (Portner 2002; Sunnucks *et al.* 2017).

Conclusion

We provide evidence that respiration of blood cells contributes significantly to whole-organism metabolism (BMR) in Eastern Yellow Robins. We estimated that *ROUTINE* respiration measured in intact blood cells with endogenous substrate supply explains ~30% of the variation in BMR. However, which exact aspect of cellular respiration drives variation in *ROUTINE* respiration remains unanswered. Nonetheless, with this newly gained knowledge and insights from previous studies we show the relevance of environmental temperatures to BMR: EYR that experienced a wider range of temperatures had lower BMR. Our data point towards a mechanism of climate adaptation independent of mitochondrial function and body mass, because the temperature experienced by EYR did not show correlation to these traits, but nonetheless BMR was explained by temperature variation.

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Appendix

Figures

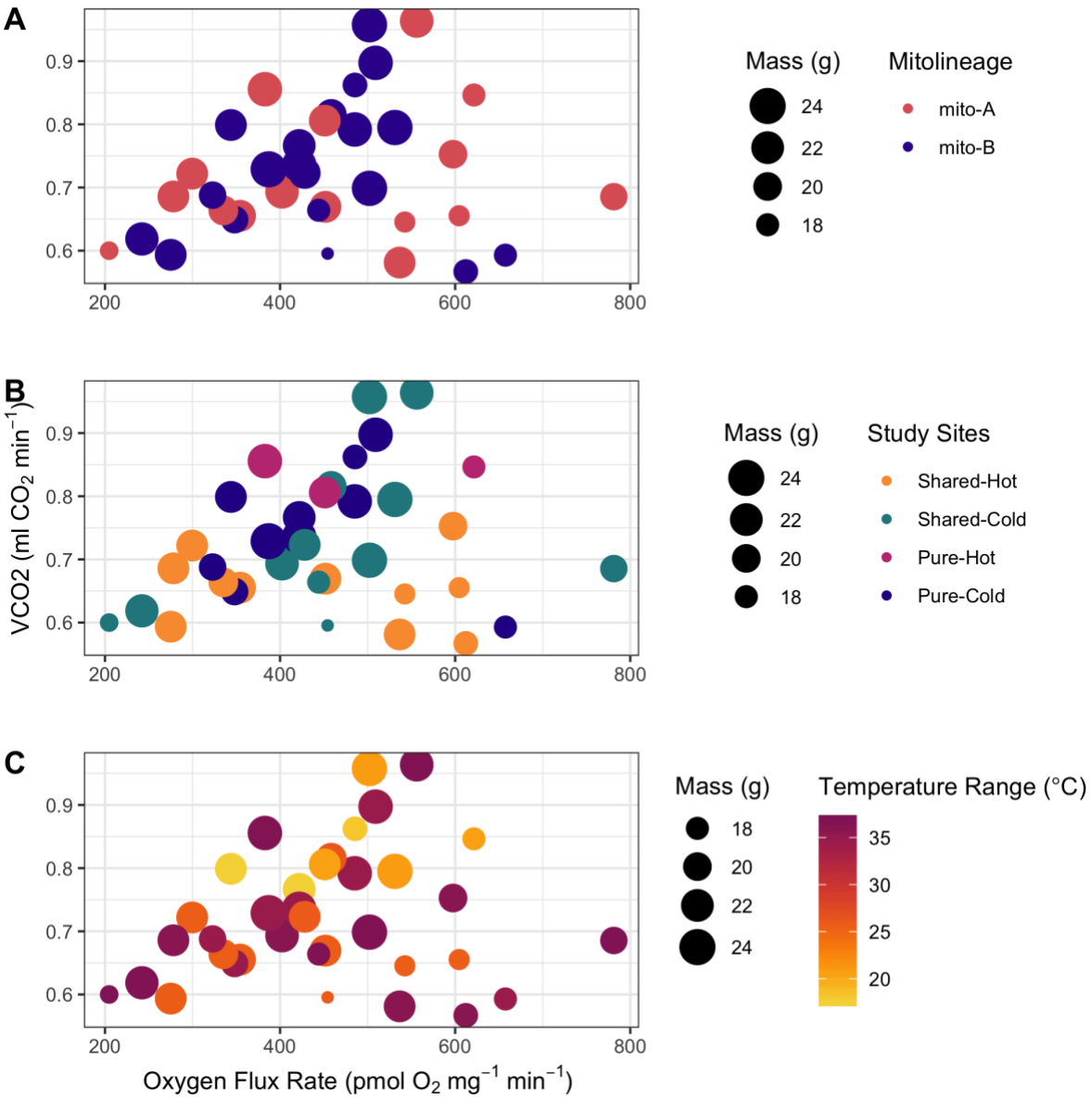

Figure S1: Relationship between mitochondrial ROUTINE respiration of intact blood cells and basal metabolic rate of Eastern Yellow Robins. Colour indicates A) mitolineage membership, B) study site, C) temperature range experienced by EYR in the 56 days prior to metabolic assessment (Table 4). The diameters of points indicate body mass (g).

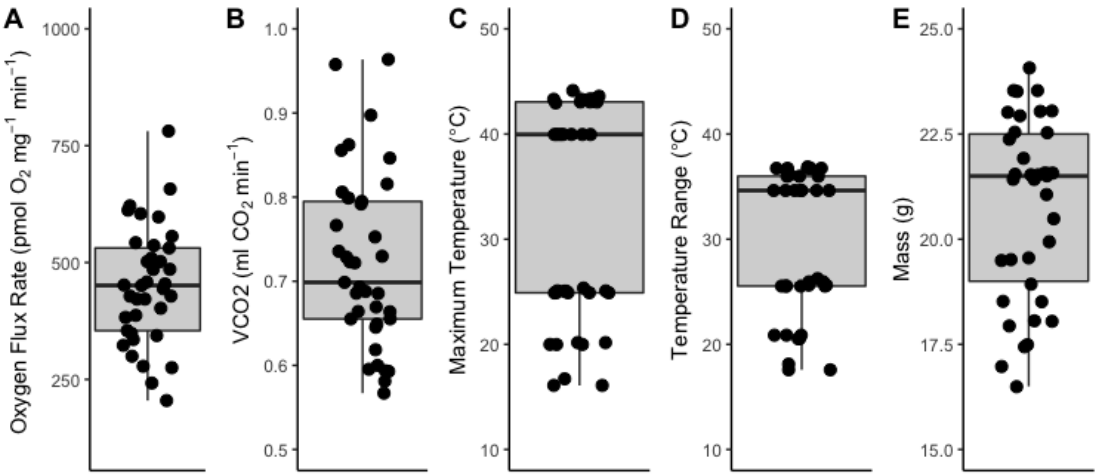

Figure S2: Distribution of dependent and independent variables previously identified as relevant for structural equation modelling of metabolism in Eastern Yellow robin (EYR). A) Mitochondrial respiration at state ROUTINE, B) whole-organism basal metabolic rate, C) maximum temperature experienced by wild EYR in the 56 days prior to measurement of the metabolic rates, and D) temperature range experienced by wild EYR in the 56 days prior to measurement of the metabolic rates, and E) body mass of EYR measured prior to metabolic assessments. B-D display non-normal distributions, likely the cause of non-normality of residuals of the models trying to link mitochondrial respiration parameters to BMR.

Tables

Table S1: Parameter estimates of the BMR model based on Table 4, where temperature range was replaced with maximum temperature.

Term	Estimate	Std. Error	t value	p value
Intercept	0.000	0.135	0.000	1.000
Mass	0.547	0.141	3.882	0.000
Maximum Temperature (56d)	-0.318	0.138	-2.309	0.027
ROUTINE (corrected for protein concentration)	0.295	0.140	2.104	0.043

Table S2: Parameter estimates of the BMR model based on Table 4, where temperature range was replaced with maximum temperature.

Term	Estimate	Std. Error	t value	p value
Intercept	0.000	0.139	0.000	1.000
Mass	0.549	0.145	3.779	0.001
Minimum temperature (56d)	-0.259	0.143	-1.815	0.079
ROUTINE (corrected for protein concentration)	0.299	0.144	2.073	0.046

CHAPTER 5

General Discussion

Mitochondria and metabolism:
towards establishing genotype-phenotype links
in climate adaptation

Stephanie Falk

This work strived to clarify the role of mitochondria in climate adaptation in the Eastern Yellow Robin. We found support for the involvement of mitochondria in shaping metabolic responses potentially relevant for climate adaptation via two paths; (1) mitochondrial function affected basal metabolism of the whole organism positively, and (2) the mitochondrial lineages differed, according to our expectations, in their metabolism in autumn, with the putatively hot adapted lineage showing a lower basal metabolic rate. These insights were gained by exploring the relationships of metabolic phenotypes of the cell (Chapter 2) and the whole organism (Chapter 3) in two geographically-distinct and climate-correlated mitolineages. Inclusion of climate and acclimation-relevant variables (weather) enabled us to infer drivers of metabolism at the cellular and whole-organism level. Finally, we established a relationship between cellular and whole-organism metabolic rate (Chapter 4). This connection indicates that cellular mitochondria-linked energy processes could shape whole-organism metabolism and thus climate adaptation in the Eastern Yellow Robin.

Contributions to the understanding of the role of mitochondria in climate adaptation

Our findings provide support for aspects of the framework of climate-driven metabolic adaptation outlined in Sunnucks *et al.* (Sunnucks *et al.* 2017). The hypothesis that mitochondrial function affects whole-organism metabolism was strongly supported. However, there was no support for the expectation that mitochondrial genetic variation is the basis of variation in mitochondrial function over and above that associated with the strong geographical association of mitolineage distribution with climate variation. We included a range of climate and weather variables (e.g. temperature, humidity, and precipitation) when testing aspects of this framework, to account for the different environmental pressures that the populations in the wild experienced. Climate and weather showed strong associations with mitochondrial function. Whole-organism metabolism was predicted by intrinsic as well as extrinsic factors: weather, season, mitochondrial function and mitochondrial genetic variation (Figure 1). Surprisingly, climate and weather variables related to humidity showed the strongest effects on cellular and whole-organism metabolism, despite the large body of research emphasizing the role of temperature in this context (Tieleman *et al.* 2003b; McKechnie 2008). These findings emphasize that metabolism will be influenced by a multitude of environmental factors and care must be taken not to oversimplify natural systems, with research only focussing on one factor.

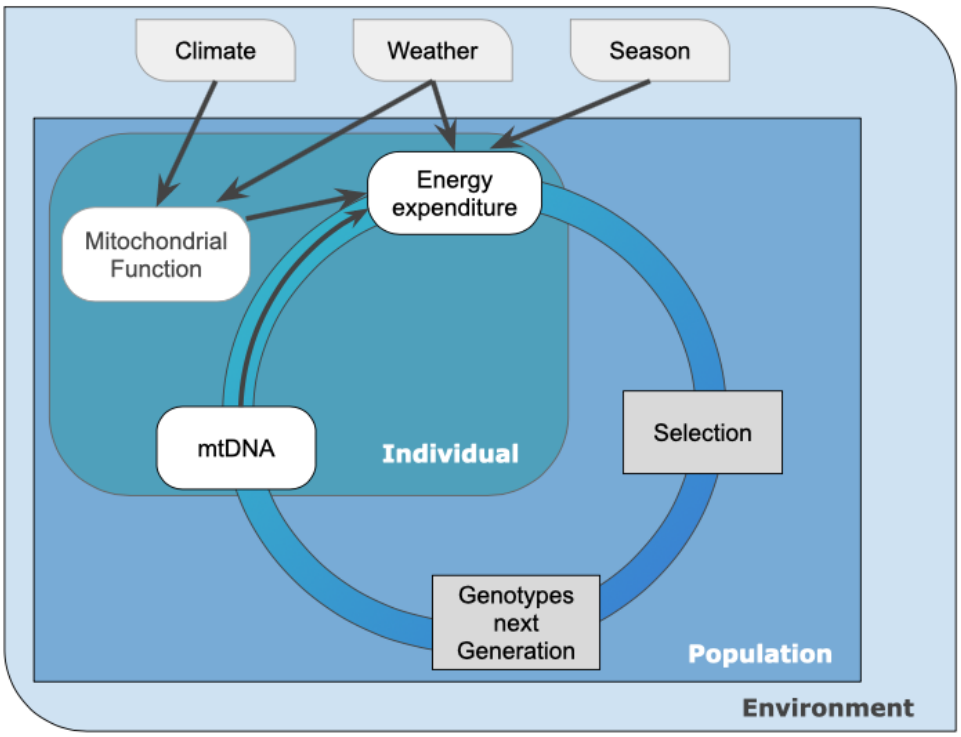

Figure 1: The investigated framework of mitochondria-mediated climate adaptation (based on Sunnucks et al. 2017) parts of which were tested in this thesis on the Eastern Yellow Robin. The arrows indicate effects identified in this thesis. We expected, but did not find evidence that mitochondrial genetic variation (mtDNA) causes differences in mitochondrial function, having accounted the strong associations of the mitolineages with different environments. We further hypothesized, but did not test, that that metabolic rate of the individual is a trait on which selection can act. This was followed by the expectation that at the population level, individuals of different metabolic rate differentially contribute to the genotypes of the next generation, which would close the circle of mitochondria-mediated climate adaptation, by determining the mitochondrial genetic variation found in individuals of the new generation. In chapters 3 and 4 we provide evidence that mitochondrial function is affected by climate and weather, and that energy expenditure is dependent on mitochondrial function and mitochondrial genetic variation, as well as weather and seasonal variation.

Alternative drivers of variation in mitochondrial and whole-organism metabolism

In the Eastern Yellow Robin, we focussed on climatic variables as predictors of local adaptation in mitochondrial function and whole organism-metabolism. However, many other environmental variables are strongly associated with climate and could explain mitolineage distribution and thus metabolic adaptation. The most relevant for energy metabolism is food abundance and nutrient availability. Environments with low food resources reduce fitness-related factors such as reproductive investment and body mass in the Eastern Yellow Robin (Zanette *et al.* 2000). In more arid and hotter environments, typically with scarcer food, more

efficient use of nutrients (that is, more efficient mitochondrial metabolism) is beneficial because less unwanted metabolic heat is produced and more useable energy is produced from the limited food resources (Sunnucks *et al.* 2017).

Another important aspect relevant to local adaptation of mitochondrial function is oxidative stress from reactive oxygen species (ROS) released as a normal product of mitochondrial function (Boveris *et al.* 1976). ROS has many regulatory roles; however, relatively high concentrations have been linked to shortened life spans. Thus, not having high ROS production is generally beneficial, but is linked to higher rates of metabolic heat production (Brand 2000; Brookes 2005; Finkel 2011; Lane 2011; Ray *et al.* 2012; Andreyev *et al.* 2015). In terms of climate adaptation, this could bring conflict at higher environmental temperatures, at which it could be challenging to balance the risk of hyperthermia and ROS production (Blier *et al.* 2014; Stier *et al.* 2014a; Stier *et al.* 2014b).

Future directions

The investigation of climate-adapted metabolic phenotypes at sites shared by both mitolineages did not demonstrate clearly that the mitolineages differ metabolically under identical climatic pressures. The potential reasons for a lack of strong differences between metabolic phenotypes of individuals of different mitolineages are manifold, and outlined in detail in chapters 2 and 3. To derive a definitive answer to the outstanding questions, detailed studies investigating potential causes of the convergence of metabolic phenotypes of individuals bearing the two mitolineages at shared sites are necessary. Multi-generation common garden experiments where both mitolineages are maintained under the same conditions (temperature and humidity), are able to efficiently separate genetic from plastic effects (Kawecki and Ebert 2004). This has been shown in other endotherms (Novoa *et al.* 2005; Dubois *et al.* 2016), as well as ectotherms (Fangue *et al.* 2009; Lamarre *et al.* 2009; Baris *et al.* 2016b). However, before commencing such common garden trials, it would be beneficial to investigate metabolic phenotypes of individuals living in a more extreme climates; in particular, Eastern Yellow Robins from more coastal regions. These experience cooler and more mesic conditions compared to the Pure-Cold locations investigated in this thesis, and may better represent the climate to which the mito-B lineage birds are adapted.

Investigating the contribution of the nuclear genome to metabolic phenotypes is of great relevance in studies of the Eastern Yellow Robin. However, in this thesis we focused only on

mitochondrial genetic variation for several reasons. We were interested in whether mitochondrial genetic variation alone was sufficient to produce climate-adapted metabolic phenotypes. We also knew from previous studies that in the southern parts of their distribution, Eastern Yellow robins have a largely neutral nuclear genetic structure, mainly driven by isolation-by-distance (Pavlova *et al.* 2013; Morales *et al.* 2017). Findings of genetic genomic islands of differentiation between nuclear genomes of populations bearing the different mitolineages (Morales *et al.* 2018) beckon their own investigation in the context of metabolism - mainly because these islands are enriched with genes encoding proteins of the oxidative phosphorylation pathway. During the timeline of this thesis, evidence was found that these islands of differentiation are associated with chromosomal rearrangements, specifically neo-sex chromosomes. This finding meant that the nuclear data collected required substantial re-investigation, involving two high quality genome assemblies and whole genome resequencing of dozens of known-sex individuals (Gan *et al.* submitted, and another in development).

Once the nuclear genomic architecture of the EYR is known, future studies will be able to link genome-wide markers with metabolism. Here, we have shown that mitochondrial genetic variation alone is not sufficient to predict mitochondrial function. While nuclear genetic variation alone may explain mitochondrial function in the individuals at pure sites, it would not explain the geographic correlation of mitolineages and climate. However, combined mito-nuclear genotypes could do so, if mito-nuclear incompatibilities built up between the two lineages when they were isolated (Morales *et al.* 2017). Thus, future research should try to elucidate whether specific mito-nuclear genotypes show signs of incompatibilities affecting mitochondrial function, including re-analysis of the present data. Such incompatibilities may be expressed as atypically high metabolic rates (McFarlane *et al.* 2016). As hybrids may experience lower fitness (Morales *et al.* 2018), individuals of incompatible but viable mito-nuclear genotypes might be rare, and preliminary data suggest this to be particularly true of females (Walters, unpublished Honours thesis, Monash University). This urges the need for large sample sizes in shared locations, to capture sufficient rare hybrids of interest. These future studies should continue to focus on the enzymatic activity of complex I of the oxidative phosphorylation pathway, but simultaneously try to explain why *ROUTINE* respiration was the sole predictor of BMR. Given (1) the complexity of natural systems, (2) the absence of differences between the lineages in mitochondrial function despite having a relatively large sample size (Chapter 2), and (3) low throughput of experiments due to low daily capture rates

(despite large efforts), future researchers might consider taking on the considerable logistic and regulatory challenges of captive breeding Eastern Yellow Robins under semi-natural conditions.

The final step in assessing whether mitolineages convey climate adaptation in the Eastern Yellow Robin is to determine their fitness in different climates. The ideal test would be to conduct reciprocal translocations (Kawecki and Ebert 2004); e.g. compare the fitness of mito-A (hot type) and mito-B (cold type) individuals under Pure-Cold conditions, and the equivalent in Pure-Hot environments. This would take into account different climates, but also other environmental factors that co-vary with climate. Reciprocal translocations in the Eastern Yellow Robin will entail considerable ethical, logistical and regulatory challenges (Kawecki and Ebert 2004; Weeks *et al.* 2011).

Concluding remarks

The work presented here highlights the importance of largely neglected aspects of climate adaptation, in particular the respective roles of mitochondria and humidity. Researchers and conservationists alike demand a better understanding of the processes involved in climate adaptation (Hulme 2005; Williams *et al.* 2008), because climate adaptation provides a prospective lifeline for many species. Physiological performance is a major trait that determines survival chances under climate change (Somero 2010). Our exploration of what governs core physiological activities sensitive to climate change led us to the conclusion that mitochondrial genetic variation, mitochondrial function and relatively short-term changes in humidity are drivers to be considered.

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