31° South: phenotypic flexibility in adaptive thermogenesis among conspecific populations of an arid-endemic bird - from organismal to cellular level

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ABSTRACT

In north-temperate small passerines, overwinter survival is associated with a reversibly increased maximum cold-induced metabolism (M_{sum}). This strategy may incur increased energy consumption. Therefore, species inhabiting ecosystems characterized by cold winters and low productivity (i.e., low available energy) may be precluded from displaying an increase in maximum metabolic rates. To examine whether M_{sum} is a flexible phenotype in such challenging environments, and ultimately uncover its underpinning mechanisms, we studied an arid-endemic small bird (Karoo scrub-robin) whose range spans a primary productivity and minimum temperature gradient. We measured M_{sum}, body condition, mass of thermogenic muscles and two indices of cellular aerobic capacity from populations living in three environmentally different regions. We found that M_{sum} was seasonally flexible, associated with aerobic capacity of limb muscles, but not increasing with lower temperatures, as predicted. Notwithstanding, the cold limit (temperature at which birds reached their maximum metabolic capacity) decreased in winter. These results indicate that birds from arid-zones may respond to cold conditions by altering thermosensation, rather than spending energy to produce heat in skeletal muscles.

INTRODUCTION

Temperature influences animal life at all levels of organization. It affects the efficiency and functionality of biochemical networks and organism physiological responses (Hochachka and Somero 2002) in such a pervasive way that extreme heat or cold can be lethal. Therefore, to withstand thermal changes, vertebrates living in seasonal environments can reversibly alter their physiological phenotype in a process termed phenotypic flexibility (Angilletta *et al.* 2010) (McKechnie and Swanson 2010).

At cold temperatures, homeothermic-endotherm animals (i.e.: birds and mammals) loose heat from their warm bodies to the environment. To maintain a high body temperature in cold conditions, birds principally increase the rate of heat production through shivering (Hothola 2004). While shivering thermogenesis results from cellular processes, namely the activation of energetic metabolism pathways to burn cellular fuels to power skeletal muscle contraction (Hothola 2004), it can be quantified at the organism-level as the maximum thermoregulatory metabolic capacity (M_{sum}; (Thompson 2010)). In fact, several studies showed that cold temperatures lead to increases in M_{sum} (Vezina et al. 2011) (Swanson et al. 2014a). One explanation for this flexibility in maximum metabolic capacity is the cold adaptation hypothesis, which posits that birds wintering in cold climates should have higher M_{sum} than those in warmer climates, and hence high M_{sum} is critical for overwinter survival in very cold regions (Marsh and Dawson 1989) (Swanson and Garland 2009). An alternative explanation is offered by the climate variability hypothesis (Janzen 1967), (Bozinovic and Naya 2014), which posits that broader climatic fluctuations results in wider flexibility in thermal tolerance as a means to cope with the fluctuating environmental conditions.

Several mechanisms, from whole-organism level down to the biochemical level, have been proposed to explain the high cold-induced M_{sum}: i) increase in body condition assessed as body

mass (Vézina *et al.* 2006) (Zheng et al. 2014); ii) increase in muscle mass, in particular the pectoralis muscle which is the primary thermogenic organ (Vézina *et al.* 2006) (Swanson et al. 2014a); iii) increase in enzymatic activity in oxidative metabolic pathways (Vézina *et al.* 2006) (Zheng *et al.* 2014) (Liknes and Swanson 2011a); and iv) increase in fat catabolism (Dawson et al. 1992) (Thompson 2010). However, evidence for flexibility in M_{sum}, as well as support for the above mentioned mechanisms driving up-regulation of M_{sum}, comes primarily from endotherms living in north-temperate ecosystems, where summer primary productivity is high (Prince and Goward 1995), and therefore individuals can afford the large energetic cost of up-regulating metabolic heat production (Hothola 2004).

In ecosystems characterized by low primary productivity and large seasonal temperature fluctuations, it is not clear how endotherms survive the low winter temperatures if not using energy saving strategies such as heterothermy. Therefore, to improve our understanding of the physiological underpinnings of adaptive thermogenesis in such ecosystems, we studied a small passerine (Karoo scrub-robin, *Cercotrichas coryphaeus*; hereafter designated scrub-robin) living in the subtropical arid-zone of southwestern Africa. Its range spans over an area of overall low primary productivity (Prince and Goward 1995) and exhibits a thermal gradient from west to east (Figure1C). As one transects from the Atlantic coast to inland, winters become increasingly colder, and primary productivity decreases as rainfall becomes increasingly unpredictable. Specifically, along the Atlantic coast winters are mild (mean minima: 8°C, record minima >0°C) and moderately productive from regular fog and predictable winter rainfall; in contrast, in the continental region minimum temperatures reach sub-zero (mean minima: -3°C, record minima <-10°C) and primary productivity is very low year-round. Given these environmental features, the scrub-robin renders an ideal system for testing whether sub-tropical birds fine-tune phenotypes associated with adaptive thermogenesis in response to local environmental conditions. We hypothesize that birds from populations experiencing sub-zero winter temperatures would

exhibit more pronounced increase of M_{sum} than populations from milder conditions, a pattern that can be driven by hypertrophy of pectoral (Swanson et al. 2014b) and lower limb (Isaac *et al.* 2014) muscles. However, low food abundance in such low productive ecosystems would hinder muscle build up. Thus, an alternative mechanism may be in place. Given the evidence showing that quantity and morphology of mitochondria (cellular powerhouses) change in response to bioenergetic cues (Putti *et al.* 2015) (Nasrallah and Horvath 2014), and that lipid droplets play an essential role in energy provisioning during exercise (Bosma 2016) and shivering in birds (Vaillancourt *et al.* 2005), we further predicted that the high metabolic capacity at organismal level would stem from increased cellular aerobic metabolism. Specifically, we anticipated that increased density of mitochondria and lipid droplets would be positively associated with M_{sum}. To test our hypothesis we developed an integrative approach at several levels of organization (Figure1).

METHODOLOGY

Study sites, sampling and ethical clearance

We selected study sites that represent three main regions along the climatic and primary productivity gradient in the scrub-robin range (Figure 1C): *Coastal, Central* and *Inland.* We captured 85 adult birds in three regions and two seasons (summer: December 2015; winter: July 2016): $n_{coastal} = 32$ ($n_{summer} = 18$; $n_{winter} = 14$), $n_{central} = 27$ ($n_{summer} = 14$; $n_{winter} = 13$) and $n_{inland} = 26$ ($n_{summer} = 10$, $n_{winter} = 16$). Birds were captured using spring traps baited with mealworms (*Tenebrio molitor*). We kept them in individual cages in a quiet room with *ad libitum* access to food for no more than 24h, when the metabolic experiments took place. We released the birds at the point of capture, except those sacrificed (details below). Each sampling location was georeferenced and the GPS coordinates were the used to extract mean minimum temperature (T_{min}) and Normalised Difference Vegetation Index (NDVI), a surrogate for primary productivity,

for the months of data collection. T_{min} was extracted from two sources: i) WorldClim V2 database (http://worldclim.org/version2; (Fick and Hijmans 2017)) which is averaged from 1970-2000 interpolated at 2.5min resolution (~5km) and ii) South African Weather Service for three weather stations closest to our study and contemporary to our experiments. NDVI at 250m resolution from USGS-LandDAAC-MODIS dataset hosted by United States Geological Survey (https://lpdaac.usgs.gov/dataset_discovery/modis).

Permits to capture, handle and sacrifice birds were issued by the Northern Cape Department of Environmental Affairs (ODB 2665 & 2666/2015; Northern Cape Province) and CapeNature (0056-AAA008-00051; Western Cape Province) in South Africa. The Animal Ethical Committee at Nelson Mandela Metropolitan University (South Africa) approved all experiments (A15-SCI-ZOO-005).

Body Condition: BM_{scaled}, fat scores and mass of thermogenic muscles

The capacity to withstand environmental challenges (known as body condition; (Hill 2011) was assessed using three indices: i) body mass scaled by size ($M_{b\text{-}scaled}$), ii) fat scores and iii) thermogenic muscles mass.

To quantify $M_{b\text{-}scaled}$, which reflects the relative size of energy reserves such as protein and fat, we used the standardization technique proposed by (Peig and Green 2009): standard major axis regression between body mass (electronic scale, d = 0.01 g) and the linear body measurement tarsus-length (calliper, d = 0.01 mm). Thus, $M_{b\text{-}scaled} = M_i \times [L_0/L_i]^{bSMA}$, where M_i and L_i are the body mass and tarsus-length of individual *i*, respectively; L_0 is the tarsus-length arithmetic mean for the study populations to which index is standardized; b_{SMA} is the scaling exponent estimated by the standardized major axis regression of mass-length.

We checked fat accumulation at the furcular depression and abdomen in all 85 adult birds, and quantified it using a scale that ranges from zero (no fat) to eight (flight muscles not visible with

fat covering the entire abdomen); (Kaiser 1993). Fat scores were verified in the birds we sacrificed to collect thermogenic muscles, as reported below.

We sacrificed 25 of the 85 birds: Coastal (n=10; 5 in each season), Central (n= 10; 5 in each season) and Inland (n= 6; summer: 1, winter: 5) by thoracic compression in the early morning after the rest-phase of birds. We excised the pectoralis and gastrocnemius muscles from the right-side body plane and measured their wet mass (electronic scale, d = 0.001 g). To estimate the total mass of pectoralis ($Pectoralis_{mass}$) and gastrocnemius ($Limb_{mass}$) we doubled the mass of the single muscles.

Aerobic capacity: immunostaining and confocal imaging of mitochondria and lipid droplets

Because mitochondria density and morphology, and lipid droplets have been shown to play an essential role in energy provisioning during exercise, we measured the density of both intra-cellular organelles in the pectoralis and gastrocnemius muscles. Immediately after weighing the muscles, we fixed them by immersion into 2% paraformaldehyde as previously described (Dahl *et al.* 2014). In the laboratory, fiber bundles were teased apart under a stereomicroscope and stored until further processing. We prepare only muscles for birds at the extremes of the environmental gradient: *Coastal* (n = 10) and *Inland* (n = 6). Mitochondria were labelled by immunofluorescence using an antibody targeting Cytochrome C Oxidase and lipid droplets were stained using an antibody targeting Perilipin2. Briefly, single muscle fibers were permeabilized, incubated with primary anti-bodies, washed, subsequently incubated with secondary anti-bodies, and finally mounted in a glass slide. We used a Zeiss LSM710 microscope (Carl Zeiss, Germany) to image 8 - 10 fibers per individual muscle, and for each fiber we collected 16-22 z-planes. Orthogonal maximal projections were obtained for the 318 fibers, which were then used for image analysis.

To estimate the area occupied by mitochondria and lipid droplets, we developed a pipeline (step-

158 by-step in Table S1, Supplementary Information) in CellProfiler Analyst (Jones *et al.* 2008). Our
159 pipeline identified nuclei area, cytoplasm area, mitochondria area and lipid droplets area of each
160 fiber. With these measures we estimated the fraction of the whole fiber occupied by mitochondria
161 or lipid droplets as follows: Mitochondria fractional area (FAMito) = Mitochondria area
162 /[cytoplasm area – nuclei area], Lipid droplets fractional area (FALipids) = Lipid droplets area
163 /[cytoplasm area – nuclei area]. Transversal mitochondrial connections were counted manually
164 from projected z-stacks. All processed images were manually curated by inspecting the overlay
165 of the objects against the original image, and troubleshoot individually as needed. For full details
166 on the immunostaining and image acquisition protocols see Supplementary Information.
167
168 ***Cold-induced metabolism, body temperature and conductance***
169 Birds showing any sign of body, wing and tail moult as well as those too agitated or not feeding
170 enough (showing mass loss exceeding 5%) were excluded from cold-exposure experiments (n =
171 18). The remaining 67 birds were fitted with temperature-sensing passive integrated transponders
172 (PIT-tag; BioThermo13, Biomark Inc., USA) to enable monitoring of body temperature (T_b)
173 throughout the experiments. Each PIT-tag was injected into the bird's abdominal cavity as
174 described in (Oswald *et al.* 2018).
175 After PIT-tag implantation, the bird rested in the cage for at least 30 min before cold-exposure
176 experiments. Cold exposure experiments took place within 24h of capture at each field site,
177 during the active phase of the birds (9am-4pm) after food was withheld for 2h.
178 To quantify metabolic rates under cold conditions, we used an open-flow respirometry system
179 (FoxBox-C Field Gas Analysis System, Sable Systems, USA) to measure bird´s O_2 consumption
180 and CO_2 production while exposed to a HelOx atmosphere (79% Helium + 21% Oxygen). HelOx
181 was used because it allows maximum rates of heat loss at higher temperatures than normal air,
182 and therefore prevents frostbite (Holloway and Geiser 2015). Data were recorded using

183 EXPEDATA software (Sable Systems). Specifically, HelOx was pushed through a respirometry
184 chamber (22 cm x 15 cm x 12 cm) holding the bird at flow rates of ~1.5 L min^{-1} using a mass
185 flow controller (Omega, USA). The excurrent air from the respirometry chamber then passed
186 through a multiplexer (Sable Systems), into an open manifold system, and was subsequently
187 pulled through the gas analysers by the FoxBox system at a flow rate of around 0.5 L min^{-1}.
188 The experiment involved placing the respirometry chamber housing the bird into a 40 L
189 fridge/freezer (ARB, Australia) serving as an environmental chamber (Noakes et al. 2017). For
190 the first 10-20 minutes of the trial, atmospheric air was pushed through the chamber (flow-rate of
191 ~1.5 L min^{-1}) to allow the bird to calm down. Trials started at air temperature raging from 15 - 20
192 °C (higher in summer than in winter) by switching the air stream to HelOx. The birds were then
193 exposed to a sliding cold exposure protocol, by reducing air temperature by 3 °C every 10
194 minutes. Ambient temperature was recorded manually every 2 min using a Cu-Cn thermocouple
195 (IT-18, Physitemp Instruments, USA) and temperature recorder (RDXL 12SD, Omega). Body
196 temperature of birds was recorded at 1 sec intervals by placing the PIT-tag reader inside the
197 environmental chamber. Trials ended when i) $\dot{V}_{CO_2}$ started to decline indicating peak thermogenic
198 metabolism had been reached, and ii) T_b < 34 °C. Unlike previously published studies on
199 passerines undergoing cold exposure that were terminated when birds reached T_b < 37 °C
200 (Swanson et al. 1996) we found that many scrub-robins showed a decline in T_b well below 37 °C,
201 while still increasing resting metabolism and hence the reason for establishing the T_b < 34 °C
202 threshold.
203 Cold exposure experiments generally took less than 1 h and peak metabolic rates were typically
204 reached within 20 minutes of HelOx. At the end of each experiment the bird was placed back in
205 the holding cage, in a warm place, with food and water available ad libitum. For each bird, we
206 recorded the time, air temperature and T_b at which M_{sum} was reached. The air temperature at

which M_{sum} was reached is defined here as the cold limit (Tc_L). To calculate M_{sum}, we obtained the highest 5 min mean $\dot{V}_{CO_2}$ during cold exposure. We chose $\dot{V}_{CO_2}$ over O_2 consumption, as our field set up did not allow for systematic drift correction in oxygen values in all individuals. In addition, we estimated thermal conductance by calculating the rate of heat loss when birds reach their M_{sum} using the following equation $C = M_{sum}/(T_b-T_a)$, where T_b and T_a represent body temperature and HelOx temperature measured within five minutes of the birds reaching M_{sum}, respectively.

Statistics

We extracted NDVI and T_{min} values for our sampling sites using the R *Raster* package (Hijmans 2017). For all variables, we tested for normality and homogeneity of variance using Shapiro–Wilk's and Levene's test, respectively. If heteroscedasticity was detected, the response variable was log-transformed. We used an analysis of variance to test for sexual dimorphism. In the event that sex was not significant, we removed the variable and proceeded with pooled sexes. We tested for the role of environmental features such as T_{min} and NDVI on body condition, mass of thermogenic muscles (mass of pectoralis and gastrocnemius) and cellular aerobic capacity (density of mitochondria and lipid droplets), cold limit (T_{CL}) and thermal conductance using generalised linear models (GLMs). We opted to use T_{min} experienced during each study period as a linear predictor instead of "season" as a categorical predictor as we believe that variation amongst study site in the former metric would explain physiological responses better.

Additionally, we examined the influence of T_{min} and NDVI, body condition, size of thermogenic muscles and cellular aerobic capacity on M_{sum} using GLMs.

All statistical analyses were performed in R v3.3.2 (R Development Core Team) and plots produced with ggplot2 package (Wickham 2016). We accepted $p \leq 0.05$ as a significant

231 difference for all statistical tests.

232

233 **RESULTS**

234 Regardless of the source of T_{min} (WorldClim or SAWS) the results were consistent; therefore for
235 the sake of brevity, in the main text we present the results for T_{min} obtained from WorldClim and
236 report results with $T_{min\ SAWS}$ in Supplementary Information.

237

238 ***Body condition: BM_{scaled}, fat depots and muscle mass***

239 We found that minimum temperature (T_{min}), but not primary productivity (NDVI), was a
240 significant predictor of size corrected body mass ($M_{b\text{-}scaled}$). Birds significantly increased body
241 condition as T_{min} decreased (GLM; $t_{Tmin} = -3.666$, $p < 0.01$); FIGURE 2A-B). At the regional
242 level, the decrease of T_{min} and NDVI lead to a significant increase of $M_{b\text{-}scaled}$ for birds living in
243 the *Inland* (GLM, $t_{Tmin} = 2.254$ p=0.03; $t_{NDVI} = 3.229$, $p < 0.01$) and *Coastal* (GLM, $t_{Tmin} = -2.763$,
244 $p = 0.01$; $t_{NDVI} = -2.506$, $p = 0.02$) regions, although no change was observed for *Central* region
245 birds.

246 Minimum temperature, but not NDVI, was significantly associated with fat deposits (GLM, t_{Tmin}
247 $= -14.795$, $p < 0.01$; FIGURE 2C-D). The amount of visible fat significantly increased as T_{min}
248 decreased in *Coastal* (GLM, $t_{Tmin} = -4.295$, $p < 0.01$), *Central* (GLM, $t_{Tmin} = -6.245$, $p < 0.01$) and
249 *Inland* (GLM, $t_{Tmin} = -2.421$, $p = 0.02$) regions. For all populations fat depots were absent to very
250 low during the summer (Fat scores = 0 - 0.25) compared to winter values ranging from 0.5 to 1.5
251 (1.5 = furcular depression almost completely covered with fat, plus small stripes of fat in
252 abdomen).

253 Overall, the pectoral muscles (n = 15, mean ± SE = 1.114 ± 0.034g x 2 = 2.228 ± 0.068 g)
254 accounted for 12.3% of scrub-robin body mass (n = 15, mean ± SE: 18.8g ± 1.382 g), while the
255 gastrocnemius (n = 15, mean ± SE: 0.089 ± 0.002 g x 2 = 0.178 ± 0.004 g) represented 0.94% of

256 birds' body mass. There was no association of $Pectoral_{mass}$ or $Limb_{mass}$ with T_{min} or NDVI:
257 $GLM_{pectoral}$, $t_{Tmin} = -0.832$, $t_{NDVI} = -1.116$, $p > 0.10$; GLM_{limb}, $t_{Tmin} = -0.832$, $t_{NDVI} = -1.116$, $p >$
258 0.10.

259

260 ***Aerobic capacity: Mitochondria and lipid droplets density***

261 Confocal micrographs of the pectoralis muscle revealed densely packed round mitochondria
262 (Figure3A). Mitochondria fractional area (FAMito) ranged from 0.615 (± 0.084) in pectoral
263 fibers, to 0.416 (± 0.092) in limb (Table S2, Supplementary Information). $FAMito_{pectoral}$ was
264 approx. 20% significantly larger than $FAMito_{limb}$ ($F = 38.054$, $p < 0.01$).
265 $FAMito_{pectoral}$ was not associated with T_{min} (GLM, $t = 2.071$, $p = 0.06$) or NDVI (GLM, $t = -$
266 0.287, $p > 0.10$). $FAMito_{limb}$ was not associated with NDVI (GLM, $t = 1.803$, $p= 0.096$) but
267 associated with T_{min} (GLM, $t = 2.994$, $p = 0.01$): mitochondria densities increased with T_{min}.
268 Transversal connections between intermyofibrillar mitochondrial networks were exclusively
269 observed in limb fibers (Figure3A). In the *inland* population, 80% of the individuals showed
270 connections, while in the *coastal* population connections were only present in 40% of the birds.
271 The mean fractional area occupied by lipid droplets (FALipids) ranged from 0.035 in pectoral
272 fibers to 0.040 in limb (Table S2, Supplementary Information), with no significant difference
273 between them ($F = 0.195$, $p = 0.66$). $FALipid_{limb}$ was significantly associated with $T_{min,}$ but not
274 with NDVI (GLM, $t_{Tmin} = 1.794$, $p = 0.04$; $t_{NDVI} = -0.617$ $p > 0.1$), showing an increase at higher
275 temperatures (Figure3B). $FALipid_{pectoral}$ did not change with T_{min} or NDVI (GLM, $t_{Tmin} = 1.082$,
276 $t_{NDVI} = 0.486$, $p > 0.1$).

277

278 ***M_{sum} association with environmental features, body condition, thermogenic organs, and***
279 ***aerobic capacity***

We observed no sexual dimorphism in maximum thermoregulatory metabolic capacity (M_{sum}; ANOVA, $F = 0.202$, $p > 0.1$). Maximum thermogenic capacity (M_{sum}) ranged from 1.373 to 3.309 mL CO_2/min, with mean = 2.341 mL CO_2/min ± 0.384 SD. M_{sum} was significantly positively related with T_{min} (GLM, $t = 2.764$, $p < 0.01$) and $M_{b\text{-}scaled}$ (GLM, $t = 2.703$, $p < 0.01$) but not with NDVI (GLM, $t = 1.044$, $p > 0.1$).

When restricting the analysis to regional-level, we found that M_{sum} increased alongside with increasing T_{min} (i.e. summer) in *Coastal* (GLM, $t = 2.903$, $p = 0.01$) and *Central* (GLM, $t = 2.577$, $p = 0.02$) populations alongside increasing T_{min} (i.e. summer), whereas birds from *Inland* showed no significant changes (GLM, $t = 1.446$, $p = 0.163$; Figure2E).

At maximum thermogenic capacity, scrub-robins defended similar body temperatures regardless of local T_{min} (GLM; $t = 1.510$, $p > 0.1$)**.** Nevertheless, the temperature that triggered the maximum heat production **-** cold limit (Tc_L) - decreased at lower T_{min} (GLM, $t = 7.661$, $p < 0.01$; Figure 2F). This association that was significant for the three regions: *Coastal* (GLM, $t = 2.523$, $p = 0.019$), *Central* (GLM, $t = 2.634$, $p = 0.017$) and *Inland* (GLM, $t = 5.034$, $p < 0.01$).

Overall, the rate of metabolic heat loss was positively associated with increasing T_{min} (GLM; $t = 4.858$, $p < 0.01$) but not with $M_{b\text{-}scaled}$ (GLM; $t = 1.471$, $p > 0.1$). Within-region, thermal conductance significantly increased with increasing T_{min} in *Coastal* and *Central* populations (GLM; $t_{Coastal} = 3.966$, $p < 0.01$; $t_{Central} = 4.976$, $p < 0.01$), while no association with T_{min} was found for *Inland* birds (GLM; $t_{Inland} = 1.962$, $p = 0.064$).

We found M_{sum} to be associated with increasing T_{min} but not NDVI (Table 1, model A). At the whole-organism level, M_{sum} significantly increased with $M_{b\text{-}scaled}$ (Table 1, model B), and decreased with fat depots (Table 1, model B). At muscular level, M_{sum} was not affected by mass of thermogenic muscles (Table 1, model C). At the cellular level, none of the proxies of aerobic capacity was associated with M_{sum} (Table 1, model D).

DISCUSSION

Cold temperatures have long been recognized as an inescapable physiological stressor for small-bodied endotherms such as birds (Tattersall *et al.* 2012). While small birds from north-temperate habitats deal with this challenge through flexibility in thermoregulatory physiological traits (e.g.: (Dawson and Olson 2003), (Vézina *et al.* 2006)), there is a paucity of information on whether sub-tropical species use similar mechanisms (Smit and McKechnie 2010), as well as whether intra-specific variation allows populations to acclimatize to local conditions (van de Ven et al. 2013).

By combining evidence from different levels of organization from populations of a small passerine living in different temperature and primary productivity conditions we found that: i) body condition increased with lower minimum temperatures (T_{min}); ii) thermogenic mass remained unchanged regardless of T_{min} or variation in primary productivity (NDVI); iii) in pectoralis muscle, density of mitochondria and lipid droplets was maintained seasonally; iv) in limb muscles, both the mitochondrial and lipid droplet densities decreased with lower T_{min}; v) at maximum thermogenic capacity, the environmental temperature that triggered maximum heat production - cold limit (Tc_L) - decreased at lower T_{min} for the three populations; nevertheless, the rate of heat loss remained unchanged for birds experiencing the wider thermal fluctuations - *Inland* birds; vi) maximum thermogenic capacity (M_{sum}) showed no seasonal flexibility in the population living in the coldest and least productive area (*Inland*: $T_{min} < 0$ °C and NDVI = 0.30), and surprisingly increased, in summer, in populations experiencing milder conditions (*Coastal* and *Central* regions) and, vii) M_{sum} was not associated with aerobic capacity in the small limb muscles or in large thermogenic pectoralis.

Overall, these results indicate that at least this arid-zone sub-tropical small bird species does not conform to the cold-adaptation hypothesis or to the climate variability hypothesis. Specifically, the birds that concomitantly experience the coldest conditions and the widest thermal fluctuations

showed the least degree of phenotypic flexibility in all eco-physiological traits quantified. Thus, our findings suggest that high M_{sum} and reduced thermal conductance is not the critical mechanism for successful overwintering in the cold winters of the arid sub-tropical habitats.

Thermogenesis in sub-tropical arid-zones

Although M_{sum} revealed to be seasonally and regionally flexible, the observed patterns contrast with our predictions. Firstly, M_{sum} flexibility was only found in populations living in the milder portion of the scrub-robin range (*Coastal* and *Central)* and not in the population exposed to sub-zero T_{min} in winter (*Inland)*. And secondly, the trend was to increase thermogenic capacity in summer, the period where M_{sum} was expected to be at an annual minimum (Swanson and Garland 2009) due to annual highest T_{min} . Our estimates of M_{sum} (assuming an respiratory exchange ratio of 0.71) are, on average, 75% of the values predicted by Swanson and Bozinovic (2011) for a 19 g oscines passerine, suggesting that M_{sum} is not directly linked to cold tolerance in this arid-zone passerine.

Our findings contrast with cold-induced increased of M_{sum} in north-temperate passerines (e.g.: American Goldfinch *Spinus tristis* (Swanson et al. 2014b), House sparrow *Passer domesticus* (Liknes and Swanson 2011a) and are only comparable to results reported for two other southern African birds living in similar latitudes in South Africa: the red-bishop (*Euplectes orix*; (van de Ven *et al.* 2013) and the white-browed sparrow-weaver (*Plocepasser mahali;* (Noakes *et al.* 2017). Both studies found population-specific patterns in M_{sum} flexibility, with some populations increasing M_{sum} in winter (in colder sites), while others showed no seasonal change.

Although the lack of increased M_{sum} in scrub-robins at colder temperatures was unexpected, it was consistent with the unchanged mass of the major thermogenic muscles (pectoralis and gastrocnemius). In contrast to northern-temperate Passeriformes that increase the mass of metabolically active organs (Liknes and Swanson 2011b), we did not observe a cold-induced

355 hypertrophy of pectoralis or gastrocnemius in scrub-robins. Therefore, we postulate that the low
356 productivity in the arid-zone of southern Africa compared to the northern-temperate regions may
357 preclude the growth of the energetically expensive thermogenic muscles. Equally unforeseen was
358 the increased M_{sum} in summer in *Coastal* and *Central* populations. We contend the summer
359 increase in M_{sum} is not related to reproduction as these populations breed late during the Austral
360 winter/spring following predictable rainfall events. Further, the elevated M_{sum} was not related to
361 moulting as we eliminated individuals showing clear signs of moult from analyses. The fact that
362 elevated M_{sum} during summer in *Coastal* populations coincided with elevated heat loss rates,
363 suggest it may be be explained by the strong summer winds at air temperatures below 20°C,
364 typical of the western coast of south Africa (Kruger *et al.* 2010). The exposure to high wind
365 speeds can result in elevated heat loss and hence favour higher thermogenic capacities despite
366 higher T_{min} = 13 °C.
367 Stressors such as exercise, cold exposure and starvation act to trigger mitochondrial biogenesis
368 (O'Brien 2011) (Putti *et al.* 2015). Thus, we tested the role of density of mitochondria and lipid
369 droplets (energy substrate) as a possible mechanism to respond to the extreme aerobic demand of
370 shivering in the scrub-robin. Our prediction that mitochondrial density in pectoral muscle should
371 increase with lower T_{min}, as a mean to power shivering, was not supported (Figure 3B). Yet, the
372 lack of difference in mitochondrial density between *Coastal* and *Inland* birds is reconciled with
373 the lack of flexibility in M_{sum} during winter for the same regions. The significant reduction of
374 lipid droplets in limb in the *Inland* population (T_{min} = -0.4 °C) together with increased number of
375 transversal connections between mitochondria (increases energetic performance (Nasrallah and
376 Horvath 2014)), suggest a depletion of energetic reserves. However, because it did not reflect an
377 increase in thermogenic capacity, we suspect the lipid reserves may have been used to power
378 walk in search for food, in the sparse arid environment.
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Thermosensation prior to thermogenesis

Besides shivering, other mechanisms are proposed to contribute to cold conditions' tolerance: insulation capacity, locomotor activity and nonshivering thermogenesis. Modulation of insulation from the environment, measured here as flexibility in heat loss at maximum thermogenic capacity (a proxy for thermal conductance), could potentially alter bird's capacities to better control heat loss. However, our estimates, besides showing no clear regional differences in termal conductance, also revealed no seasonal differences in thermal conductance in the population experiencing the coldest temperatures (*Inland*).

Increasing locomotor activity exploits the thermodynamic inefficiency of catabolic reactions in skeletal muscles (60% energy released as heat) and nonshivering thermogenesis uses a deflection of proton flux in mitochondria from ATP production to heat production (Hochachka and Somero 2002). However, none of the mechanisms seem plausible in the scrub-robin. First, because being on the move for the sake of heat production in low productivity environments may lead to a mismatch between energetic demand and supply. And secondly, recent work suggests that nonshivering thermogenesis is not a prominent contributor to thermogenesis in adult passerines (Cheviron and Swanson 2017). With neither locomotor activity nor nonshivering thermogenesis responsible for thermoregulation, we propose the explanation may be on how the scrub-robins sense the cold. This ideas is supported by the fact we recorded a drastic reduction in T_b to 34 – 36 °C, while birds maintained their maximum metabolic capacity, and remarkably that scrub-robins could fly at ease at T_b = 30 - 34 °C, soon after the cold-experiment. It is thus possible that intra-specific variation exists in the somatosensory system (cutaneous thermoreceptors; e.g.: TPRM8 channel (Matos-Cruz *et al.* 2017)) and hence some populations tolerated prolonged exposure to cold and hypothermia without engaging in active production of heat. An understanding of the sensory perception of cold in Karoo scrub-robin, as the first system to mediate organism-environment interactions (Gracheva and Bagriantsev 2015), is certainly warranted.

To conclude, we believe our study exemplifies that only when implementing a detailed framework at the intra-specific level one can contemplate to understand the factors underlying phenotypic flexibility and ultimately its role in adaptation.

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Author contributions

AMR and BS conceived the study. AMR collected field data and conducted statistical analyses with critical input from CP, MTPG and BS. AMR and CP performed immunostaining and confocal microscopy imaging. NBP and BS collected physiological data. All authors wrote the manuscript and gave final approval for publication.

Data accessibility

Data used in this study is provided in a spreadsheet as Supplementary Information.

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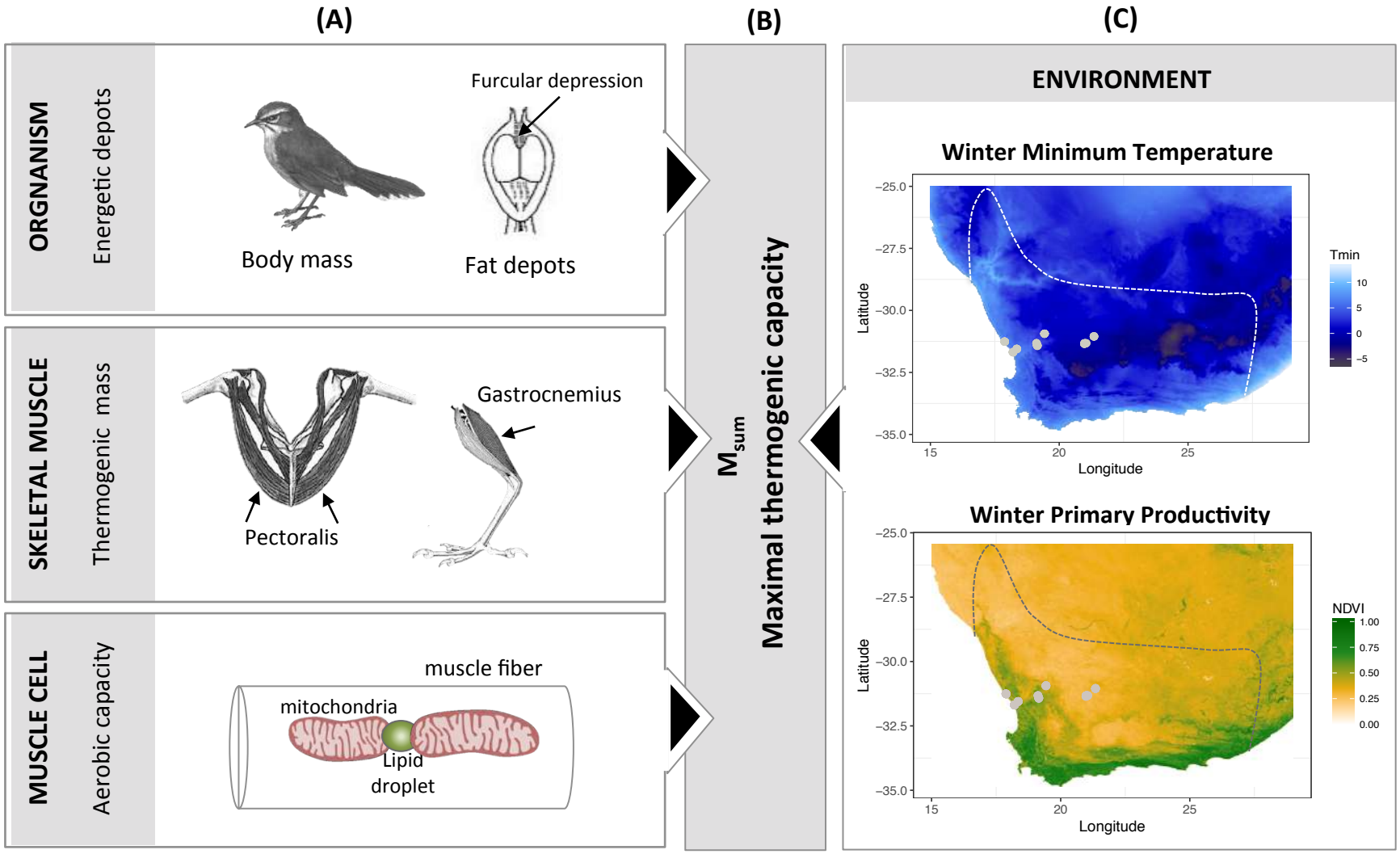

Figure1. Framework to study the mechanisms of adaptive thermogenesis in small birds living in arid-zones. (A) The underlying mechanisms driving (B) flexibility in maximal thermogenic capacity, and the role of (C) environmental variables in shaping those mechanisms. Study sites relative to the species range (delimited by a dashed line) are depicted in the maps.

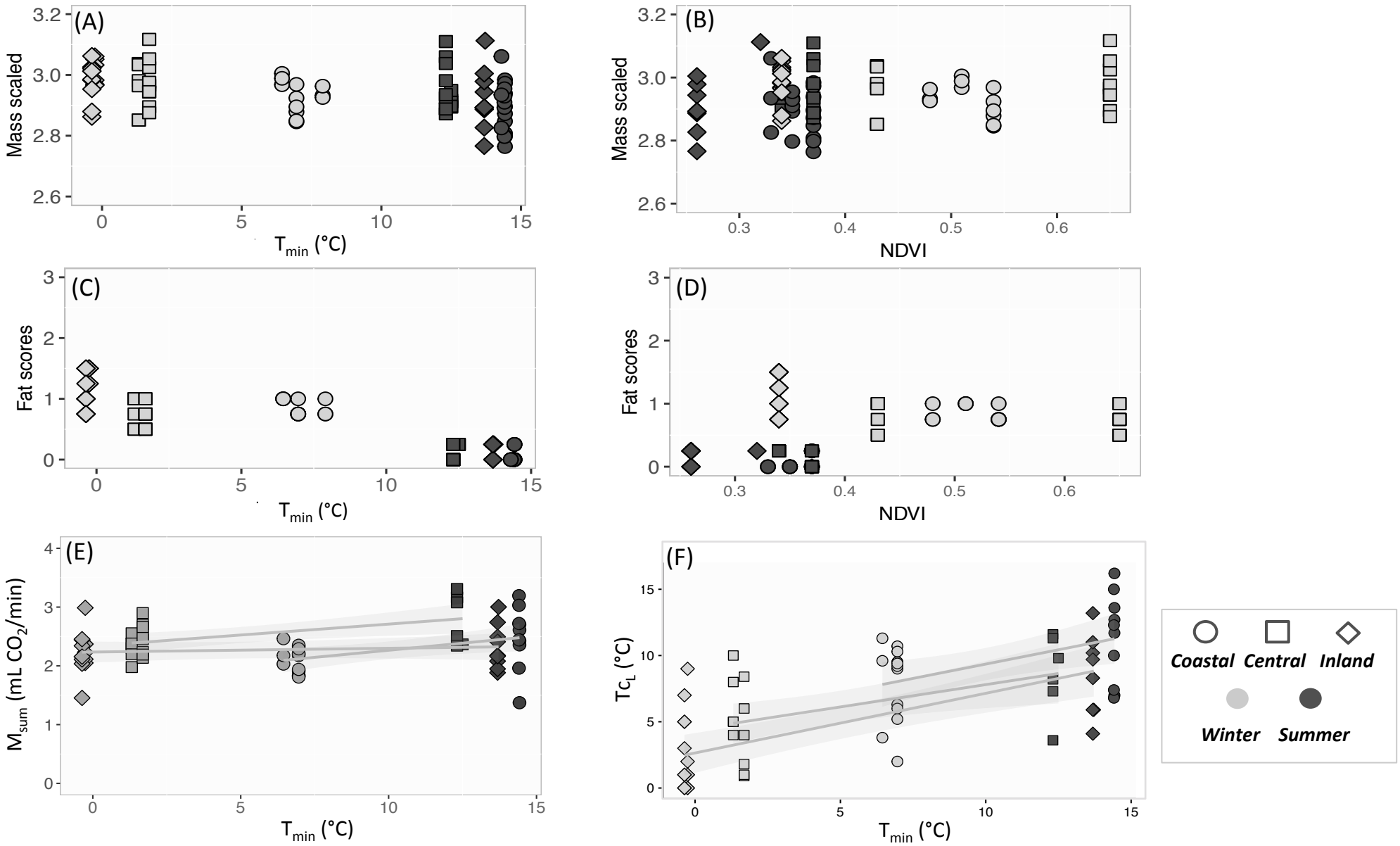

Figure2. Variation in physiological traits in response to mean minimum temperature (T_{min}) and primary productivity (NDVI). (A-B) Mass scaled, (C-D) Fat scores, (E) maximum thermogenic metabolic capacity (M_{sum}) and (F) cold limit (Tc_L).

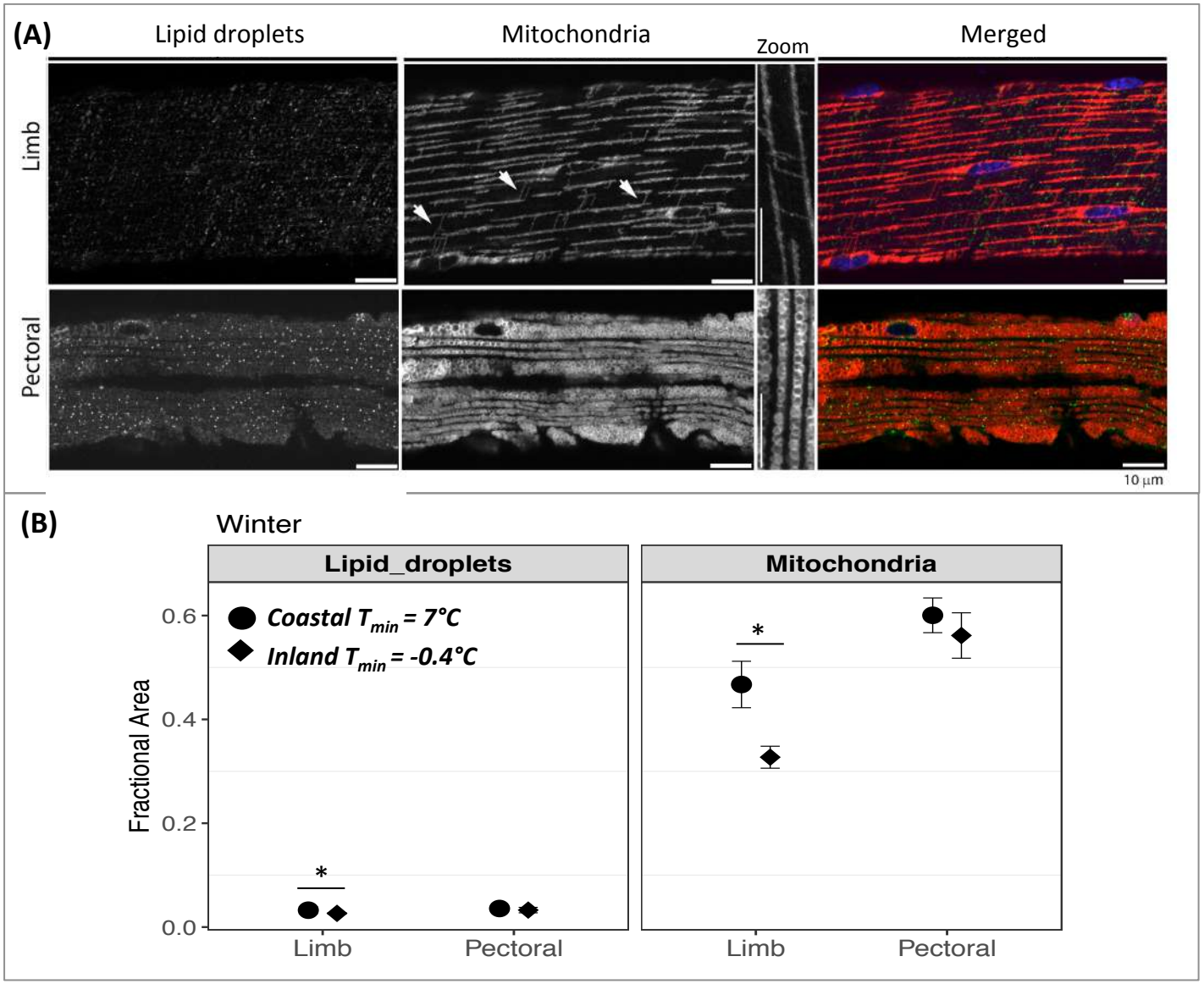

Figure3. Variation in aerobic capacity in thermogenic muscles. (A) Confocal micrographs depicting mitochondria and lipid droplets (Perilipin2). Merged image show mitochondria (red), lipid droplets (green) and nuclei (blue). Transversal connections between mitochondria, exclusive of limb fibers, are indicated with arrows and visualized in zooming in images. Bars: 10 µm. (B) Portion of muscle fiber occupied by lipid droplets and mitochondria in limb and pectoral, in winter, in *Coastal* and *Inland* populations. Significant differences ($\alpha = 0.05$) are annotated with an asterisk.